DECLARATION

I, **Rethikala P K**, hereby certify that I had personally carried out the work depicted in the thesis entitled, ***"Novel Antimicrobial and Growth Factor Releasing Hydrogel Systems For Burn Wound Management"***, except where due acknowledgment has been made in the text. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Thiruvananthapuram
30-12-2015

Rethikala PK
Reg.No: 2010/PhD/11

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES & TECHNOLOGY
BIOMEDICAL TECHNOLOGY WING, POOJAPPURA
THIRUVANANTHAPURAM – 695011, INDIA
(An Institute of National Importance under Govt. of India)
Phone-(91)0471-2520221 Fax-(91)0471-2341814 www.sctimst.ac.in

Dr. V. Kalliyana Krishnan
Scientist G (Senior Grade)
Dental Products Laboratory
BMT Wing, SCTIMST
email: kalyankv@sctimst.ac.in

This is to certify that **Ms. Rethikala P K**, in the Dental Products Laboratory of this Institute has fulfilled the requirements prescribed for the Ph. D. degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The thesis entitled, ***"Novel Antimicrobial and Growth Factor Releasing Hydrogel Systems for Burn Wound Management"*** was carried out under my direct supervision. No part of the thesis was submitted for the award of any degree or diploma prior to this date.

Thiruvananthapuram — Dr. V. Kalliyana Krishnan
30-12-2015 — (Research Supervisor)

The thesis entitled

“NOVEL ANTIMICROBIAL AND GROWTH FACTOR RELEASING HYDROGEL SYSTEMS FOR BURN WOUND MANAGEMENT”

Submitted by

Rethikala P K

for the degree of

Doctor of Philosophy

of

SREE CHITRA TIRUNAL INSTITUTE
FOR
MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM

Is evaluated and approved by

…………………………….	………………………..
Dr. V. Kalliyana Krishnan (Research Supervisor)	Examiner

Dedicated to

GOD ALMIGHTY & MY FAMILY

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ABBREVIATIONS

HEMA	: 2-hydroxyethylmethacrylate
ASTM	: American Society for Testing and Materials
TPO	: 2, 4, 6 Trimethyl Benzoyl Biphenyl Phosphine oxide
EGF	: Human recombinant epidermal growth factor
TEM	: Transmission electron microscope
FT-IR	: Fourier Transform Infra Red spectrophotometer
SEM	: Scanning electron microscope
TGA	: Thermal Gravimetric Analysis
WVTR	: Water vapor transmission rate
DSC	: Differential Scanning Calorimetry
DTA	: Differntial Thermal Analysis
ESEM	: Environmental Scanning Electron Microscopy
E	: Elongation
EtBr	: Ethidium bromide
ETO	: Ethylene Oxide
FBS	: Fetal Bovine Serum
FDA	: Food and Drug Administration
Hb	: Haemoglobin
ABAM	: Antibiotic antimicotic
HDPE	: High Density PolyEthylene
ICP/OES	: Inductively Plasma Optical Emission Spectroscopy
ISO	: International Organization for Standardization
IU	: International Unit
M	: Modulus
Mn	: Number average molecular weight
MW	: Molecular Weight
PBS	: Phosphate Buffered Saline
PCL	: Poly(ε-caprolactone)
PEG	: Poly(ethylene glycol)
pHEMA	: poly(2-hydroxyethylmethacrylate)
RBC	: Red Blood Cell
SD	: Standard Deviation
SEM	: Scanning electron microscopy

SNPs	: Silver Nano-Particles
TS	: Tensile Strength
UTM	: Universal Testing Machine
H-EGF	: Hydrogel system loaded with epidermal Growth Factor
HSN	: Hydrogel system incorporated with silver nano particles
Hdrg	: Hydrogel system incorporated with drug ofloxacin
XRD	: X-ray diffraction
ZIA	: Zone of Inhibition Assay
TPO	: 2, 4, 6 Trimethyl Benzoyl Biphenyl Phosphine oxide
DI/W	: Deionised water

SYNOPSIS

Wound is defined as disruption of cellular, anatomical, and functional continuity of a living tissue. It is caused by physical, chemical, thermal, microbial, or immunological insult to the tissue. Among these, burn wounds due to fire are considered as one of the 15 leading causes of death in India. Burn injuries are characterized by presence of non viable tissue, body fluid, serum, blood and colony of potentially pathogenic bacteria and can cause significant skin loss which may lead to mortality. As per global statistics, nearly 2, 65,000 deaths are reported to occur annually due to burn injuries in which nearly 1, 40,000 deaths occur in India alone.

Lethal problems associated with burn injuries are fluid loss as well as infection. Therefore proper wound management is required to prevent excessive fluid loss and bacterial invasion which can be achieved only by using proper wound dressing material. An ideal dressing is expected to provide a moist wound environment, absorb exudates, eliminate dead space, not harm the wound and provide a bacterial barrier while promoting wound healing. Unfortunately there is a huge dearth of affordable burn wound dressing material in India. Some imported brands are available but are very expensive and unaffordable.

Traditionally, infection of burn wounds can be controlled by the topical application of antimicrobial ointments such as silver sulphadiazine, mafenide acetate etc. But these have many limitations. So a wound dressing incorporated with an antibiotic agent may be able to control the infection as well as coverage. Similarly, growth factors also tend to play an important role in wound healing. Epidermal

growth factor (EGF) secreted by keratinocytes has been found to help in reepithelialization. The half life of EGF is too short to exert biological activity. Therefore, encapsulation of EGF in to a polymer matrix and its sustained release is expected to improve its *in vivo* efficiency.

It has been reported that wounds with a moist environment heals faster compared to wounds in a dry state. Hydrogels are a class of materials which can take up large amounts of water and possess all properties required for an ideal wound dressing material. They have the capacity to absorb wound exudates and safely retain them within the gel, which provides a microclimate that stimulates and regulates most of the cellular functions and nutritional processes during the individual phases of wound healing. They can also be easily removed from the wound surface because of their non-adherent character. Many attempts have been made earlier by various groups to use different forms of hydrogels in wound dressings. However one of the main drawbacks of hydrogel based wound dressings is their poor mechanical strength.

In this background, the main goal of the study was (1) to develop novel hydrogel based wound dressing materials with adequate strength which can provide temporary wound coverage (2) to prepare a matrix which can deliver antimicrobial / antibiotic agent to the wound site to control infection at the site and (3) to prepare a wound dressing matrix which can release growth factor to improve the wound healing effectively. The study is represented in six chapters. The background and introduction of the work are presented in Chapter 1. It explains in detail about burn wounds and state of art of burn injuries. It also details the anatomy and physiology of

skin and wound healing process, major challenges in burn wound management and classification of burn wound dressing material

Hypotheses put forward on the basis of current knowledge are: *(1) Development of a non adherent and cytocompatible hydrogel matrix based on pHEMA, using a unique photo-polymerization technique, as a wound dressing material (2) Development of an antimicrobial hydrogel matrix to control the infection at the wound site (3) Development of growth factor incorporated hydrogel matrix to promote wound healing*

Major objectives of the current study are identified as follows.

- To prepare poly(2-hydroxy ethyl methacrylate)-poly(caprolactone)-poly(ethylene glycol) [pHEMA-PCL-PEG] hydrogel matrix by photo-polymerization technique using the photo-initiator 2,4,6 trimethyl benzoyl biphenyl phosphine oxide (TPO) and their optimization
- To characterize physico-mechanical properties of pHEMA-PCL-PEG hydrogel matrix as a wound dressing material
- To fabricate and characterize antimicrobial hydrogel wound dressings using silver nanoparticle (SNP) and ofloxacin antibiotic
- To evaluate the elution profile of antimicrobial agents and their antimicrobial property
- To develop hydrogel matrix incorporated with EGF (H-EGF) and its characterization
- To evaluate surface morphology and hydrophilic character of H-EGF
- To analyze elution profile of epidermal growth factor from the matrix H-EGF and to study the effect of growth factor on fibroblast growth

In Chapter 2, exhaustive literature review has been carried out to understand the current status of burn wound dressings. The topics reviewed include burn wounds, wound healing, burn wound management using various wound dressing materials. It also reviews the importance of hydrogel based systems in burn wound management. Review also summarises the incorporation strategies of antimicrobial agents and growth factor into hydrogel systems for wound dressing applications.

In Chapter 3, experimental design in order to achieve the objectives of the proposed study is elaborated. It includes detailed description of materials employed, experimental protocols and instruments employed for the present study. Polymerisation and fabrication of hydrogel matrix is shown in Section 1. Synthesis protocols of poly(ethylene glycol) [PEG] protected silver nanoparticles (SNP) is described in Section 2. Fabrication of SNP incorporated hydrogel system is explained in Section 3. Section 4 includes fabrication of ofloxacin incorporated hydrogel systems. Preparation of epidermal growth factor incorporated hydrogel matrix is described in Section 5. FT-IR and XRD techniques were adopted for structural characterization of hydrogels. Mechanical testing using Universal Testing Machine was adopted to study mechanical properties. Methodologies adopted for the physical property evaluation such as swelling analysis, water vapour transmission rate evaluation have been described. Measurements and analysis of surface wettability using goniometer, surface morphology using Scanning Electron Microscopy (SEM) and Environment Scanning Electron Microscopy (ESEM) are also detailed. Methods of thermal analysis of hydrogel matrix using TGA and DSC are explained. Experimental details are also given for characterization of silver nanoparticle (UV-Visible spectrum and TEM), *in vitro* release profile SNP

(ICP/OES) and ofloxacin (UV spectrophotometer) and antimicrobial activity demonstration (Zone of Inhibition Assay). *In vitro* release of Epidermal Growth Factor (EGF) from the hydrogel matrix using fluorescence spectroscopy is described in detail. Cytotoxicity evaluation of hydrogel matrices using direct contact and live/dead assay, haemolytic potential of hydrogels evaluated as per ISO 10993-part 4 are provided. Details of cell adhesion studies carried out using fluorescence microscopy after staining with Texas red conjugated Phalloidin for actin filaments are explained. Tritiated thymidine uptake assay for the quantitative evaluation of fibroblast proliferation is detailed.

Chapter 4 includes results presented substantiated by figures, tables and graphs. Properties of hydrogel system containing HEMA, PCL and PEG were optimized. Out of various hydrogel systems, HI-3 (HEMA-PCL-PEG 85:10:5) which possesses better swelling and mechanical property was selected for further studies. Cytotoxic study (direct contact test and live/dead assay) and haemolytic study indicated the hydrogel to be non-cytotoxic to fibroblast cells as well as non hemolytic. Studies using actin staining showed that hydrogel matrix is non-adherent to fibroblast cells. SNP solution was synthesized and characterized using TEM and UV-VIS spectroscopy and SNP incorporated hydrogel system (HSN-3) was prepared. ICP analysis indicated 75% release of SNP from HSN-3 within 4 days. Cytotoxicity results showed SNP incorporated hydrogel (HSN-3) to be non-cytotoxic. Antimicrobial activity was confirmed using zone of inhibition assay. Photo-polymerization technique was also effectively used to prepare ofloxacin incorporated hydrogel system (Hdrg). Release kinetics was carried out after drug loading and it was found that release rate became faster with higher amount of drug

in the matrix. Direct contact test and live/dead assay using fibroblast cells confirmed the non-cytotoxic character of Hdrg. Antimicrobial activity of Hdrg was comparable to that of Ofloxacin as well as Gentamicin. Incorporation of epidermal growth factor to the hydrogel system was achieved by solvent sorption method. Fluorescence spectral data indicated nearly 100% EGF release occurred from the matrix within 48hours. Tritiated thymidine uptake assay indicated significant increase in fibroblast cell proliferation in H-EGF compared to control (cell in culture plate) and hydrogel matrix without EGF. Doubling time also decreased significantly in presence of H-EGF.

In Chapter 5, results are discussed and analyzed with the aid of current literature. It is shown that hydrogel wound dressing material with good swelling property, mechanical properties, non-cytotoxicity, non cell adherent and antibacterial property will be important criteria to achieve an ideal wound dressing material. The importance of present study has also been highlighted.

Chapter 6 summarises the results and conclusions are drawn from the present study. A hydrogel system based on pHEMA-PCL-PEG is developed successfully with potential application as a short term burn wound dressing material. pHEMA-PCL-PEG hydrogel systems incorporated with SNP and antibiotic ofloxacin respectively are expected to control infection at the burn wound site. pHEMA-PCL-PEG hydrogel incorporated with EGF is proposed for burn wounds where delayed wound healing is expected. The limitations of the current study have been identified. Citations are listed in the bibliographic section.

CHAPTER 1

INTRODUCTION

A wound is defined as a disruption of the cellular, anatomical, and functional continuity of a living tissue. It is produced by physical, chemical, thermal, microbial, or immunological insult to the tissue. Of the different types of wounds, burn wounds due to fire are considered as one of the 15 leading causes of death in India. Global statistics shows that nearly 2, 65,000 deaths occur annually due to burn injuries and of this, nearly 1,40,000 deaths occur in India alone (WHO Report, 2014).

Burn injuries are characterized by the presence of non-viable tissue, body fluid, serum, and blood and colonized by potentially pathogenic bacteria, resulting in significant skin loss, fluid loss and infection, which can lead to death. Therefore, proper wound management is required to prevent excessive fluid loss and bacterial invasion, and this can be achieved only by using appropriate wound dressing material. Traditionally, infection of burn wounds can be controlled by the topical application of antimicrobial ointments such as silver sulphadiazine, mafenide acetate, etc. However, these have many limitations. So a wound dressing incorporated with an antibiotic agent may be able to control infection better as well as provide proper coverage. An ideal dressing is one that can provide a moist wound environment, absorb exudates, eliminate dead space, does not harm the wound and provide a bacterial barrier while promoting wound healing.

Unfortunately, there is a huge dearth of affordable burn wound dressing material in India. Some imported brands are available but they are very expensive and unaffordable.

1.1.Anatomy and physiology of skin

The skin is the largest organ of the body, with a total area of about 1.8 square meters and accounting for 16% of total body weight. It contains dynamic, complex, integrated arrangement of cells, tissues and matrix elements that perform a variety of functions. It serves as an internal barrier, which protects our body from external environment, toxic pathogens and chemicals. It plays an important role in physiological function such as immune surveillance, self-healing, thermoregulation sensory detection, and much more.
(Bensouilah and Buck, 2006). The skin consists of three layers. The epidermis is the outermost layer and acts as a physical as well as chemical barrier between the external environment and the interior body and creates our skin tone. The dermis is the second layer which gives the skin its mechanical properties, elasticity and pliability. The subcutaneous layer is the third layer of the skin (Kanitakis, 2002).

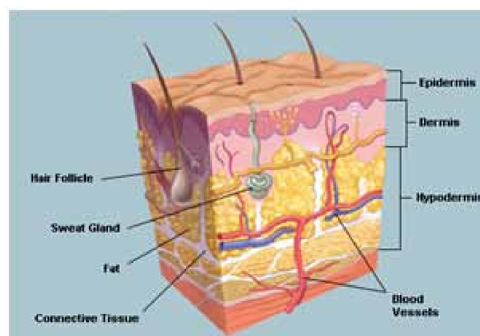

Figure 1. Schematic image representing structure and anatomy of skin(http://www.webmd.com/)

1.1.1. Epidermis

The epidermis is the relatively thin, tough outermost layer of the skin. The main cell type in the epidermis is keratinocyte, which produces the protein called keratin. The thickness of epidermis varies from 0.05 mm on the eyelids to 0.8-1.5mm on the soles of the feet and palms of the hand. While the thick epidermis of the palms and soles contain mainly five layers, it consists of four layers in other areas (Richardson M, 2003, Ro BI, 2005). The additional layer present in the thick epidermis is the *Stratum lucidum*. Moving from the lower layer upwards to the surface, the four layers of the epidermis are

- *Stratum basale* (basal or germinativum layer)
- *Stratum spinosum* (spinous or prickle cell layer)
- *Stratum granulosum* (granular cell layer)
- *Stratum lucidum*
- *Stratum corneum* (horny layer)

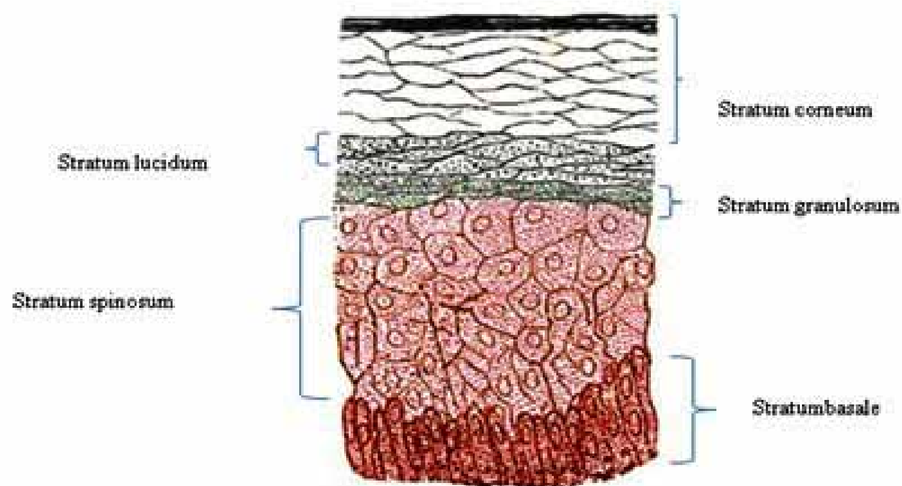

Figure 2. Layers of epidermis (https://commons.wikimedia.org/wiki/File:Piel.png)

1.1 .2. Dermis

The dermis is an integrated system of fibrous, filamentous and amorphous connective tissue that accommodates stimulus-induced entry by nerve and vascular networks, epidermal derived appendages, fibroblasts, macrophages and mast cells. The dermis comprises the bulk of the skin and provides pliability, elasticity and tensile strength. Its thickness varies from 0.6mm on the eyelids to 3mm on the back, palm and soles. It is found below the epidermis and contains mainly two layers

- *a thin papillary layer*
- *a thicker reticular layer*

The papillary dermis lies below and connects with the epidermis. It contains thin, loosely arranged collagen fibers. Thicker bundles of collagen run parallel to the skin's surface in the deeper reticular layer, which extends from the base of the papillary layer to the subcutis tissue.

1.1.3. Subcutaneous fat layer

The subcutaneous layer is the innermost layer of the skin which mainly comprises fat tissue. The layer provides a linkage between the outer skin layers and underlying structures. The fatty layer acts as a thermal barrier and protects our body from mechanical injury.

1.2 Classification of burn wounds

Burn wounds are classified according to the depth of the layers affected

(1) *Superficial burns*: affect the epidermis, characterized by a reddish appearance; heal within 3-5 days without scarring, for example, sun burn

(2) *Partial thickness burn*: the epidermis and dermis are affected, needs extensive time to heal

(3) *Full thickness burns*: the epidermis, dermis and subcutaneous layers are affected, white yellow, leathery appearance, no pain when touched

(4) *Fourth degree burns*: all the skin layers and muscles are affected, loss of function, black or charred appearance, may require amputation

1.3. Wound healing

Wound healing is a specific biologic process in which an injured tissue is repaired. The wound healing process involves four overlapping phases: hemostasis, inflammation, proliferation and remodeling (Lorenz *et al.*, 2000 and David K *et al.,* 1999)

1.3.1. Hemostasis

Hemostasis is the first stage of wound healing. It happens within 30 minutes of injury. Soon after the injury occurs, blood vessels in the wound site contract to prevent excessive bleeding and this is followed by the formation of a stable clot by the interaction of platelets and fibrin in the blood. The fibrin clot stops bleeding from the wound. Platelets are the major cell type involved in the phase which secretes the platelet-derived growth factor (PDG), an important cytokine involved in the initiation of subsequent stages of wound healing (Martin , 1997).

1.3.2. Inflammation

The inflammatory phase is characterized by the erythema (redness), swelling and warmth in the surrounding area of the injured site and this stage lasts for 4 days. In this phase, the wound is sterilized by the action of leukocytes. Increased vascular permeability allows

the infiltration of neutrophils and monocytes into the injured area. While the neutrophils scavenge the cellular debri, bacteria and foreign bodies at the wound, the monocytes differentiate into macrophages. Macrophages help in phagocytosis of the cellular debri and bacteria and secrete growth factors. The growth factors activate and attract fibroblasts, local endothelial cells and keratinocytes.

1.3.3. Proliferation

The proliferative phase is characterized by three major events (1) reepithelialization: restoration of the basement membrane zone (2) angiogenesis: establishment of appropriate blood supply (3) fibroblasia: reinforcement of the injured dermal tissue. This phase may take 2 to 3 weeks to complete, depending upon the nature of wound.

1.3.4. Remodeling phase

The remodeling phase begins about 2 to 3 weeks after the injury and can last for years depending up on the type of injury. During the remodeling phase, the strength of skin progressively increases and reaches 80% of original skin strength. Fibroblasts play key role in the process.

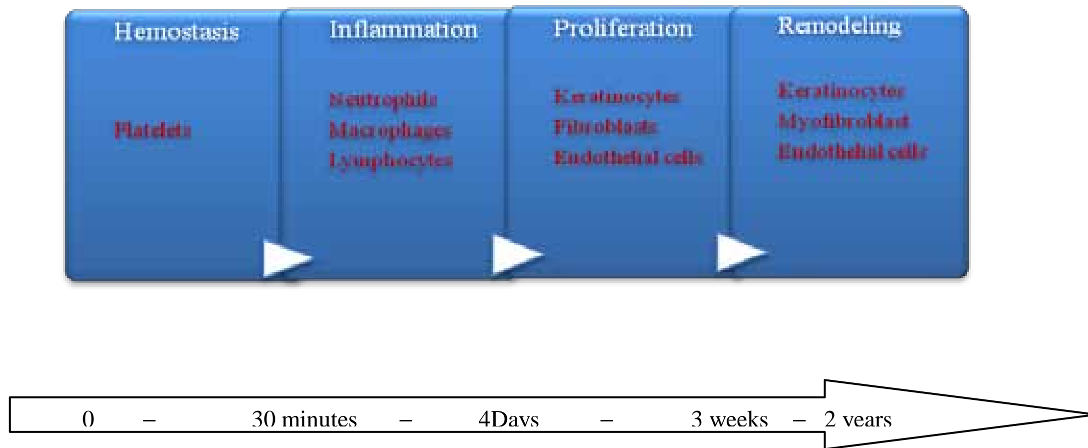

Figure 3. Wound healing phases

1.4. Burn Wound management

The main objective of wound management is to provide protection in the absence of the body's protective layer. Burn wounds are considerably complicated compared to other types of wounds. Major challenges in burn wound management are (1) Fluid loss: there is excessive fluid loss from patient's body within 24 h of injury which may lead to fall of body temperature and increased metabolic rate (2) Infection: this may delay healing and organ failure (3) Lack of uniformity of the wound: difficulty in proper coverage may cause non-uniformity in healing. Proper wound management should be carried out to prevent excessive fluid loss and bacterial invasion and can be achieved only by covering the wound using wound dressing material (Dagalkis *et al*., 1980, Matsuda K *et al*., 1992). The extent of the burn area should be measured and the wound has to be cleansed to remove debris and foreign contaminants prior to covering the wound. Estimation of the burn area is critical in the initial stage of wound management. Generally, the extent of the

burn area is expressed as percentage of total body surface area (TBSA). The wound area has to be covered using a wound dressing material to protect it from fluid loss and bacterial invasion.

1.4.1. Assessment of burn area

There is large loss of body fluid immediately after burn injury. To compensate the fluid loss from the body, fluid resuscitation has to be done clinically if the total burn surface area (TBSA) is greater than 15% and 10% of the total body surface area (TSA) in adults and children, respectively (Hettiaratchy *et al* , 2005). Three methods are used to calculate the extent of the burn area: (1) Palmar surface (2) Wallace rule of nine (3) Lund and Browder chart.

1.4.1.1. Palmar surface

The surface area of a patient's palm accounts for 0.8% of the total surface area. This method can be used to estimate very small as well as very large burns, but is not applicable to medium-sized burns.

1.4.1.2 Wallace rule of nine

In this method, the patient's body is divided into multiples of nine. The Wallace rule of nine is a good and easy way to estimate the extent of burn area in adults.

1.4.1.3 Lund and Browder chart

The Lund and Browder chart is applicable to both children and adults. Since it compensates for the variation in body shape with age, the chart provides an accurate idea of the burned body surface area in children.

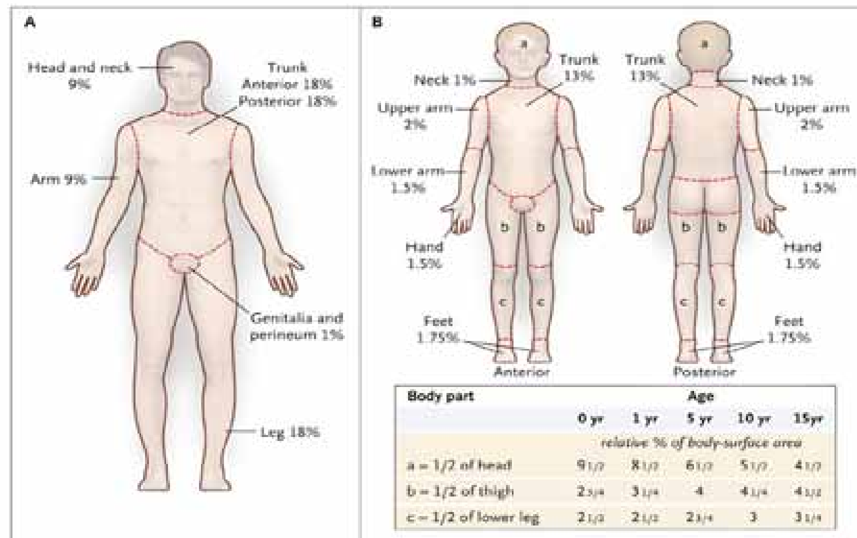

Figure 4. (A) Wallace rule of Nines (B) Lund and Browder chart

(Reference: Orgill DN Engl J Med 2009;360: 893-901)

1.4.2. Burn Wound Dressings

The primary goal of wound dressing material is to protect the wound from the external environment while simultaneously promoting the wound healing process. The major problems as far as a burn wound is concerned are fluid loss and infection. So an ideal dressing material need to be used for the protection of the burn wound. The characteristic properties of an ideal wound dressing material are as follows:

- Maintains a moist wound environment
- Absorb exudates
- Eliminates dead space
- Provides a bacterial barrier
- Promotes wound healing.

- Nontoxic – biocompatible
- Non-adherent to the wound
- Cost-effective

1.5. Classification of Burn Wound Dressings

There are different classes of burn wound dressings based on their function in the wound (Turner *et al*., 2000), type of material used in the preparation of the dressing (Queen *et al,* 2004) and the physical form of the dressing (Falabella , 1999). Wound dressings are also classified on the basis of their function as primary, secondary and island dressings (van Rijswijk, 1999). A primary dressing comes in contact with the wound surface. Secondary dressings cover the primary dressing to achieve protection effectively, while the island dressing consists of an absorbent pad and adhesive backing and absorbs the excess wound fluid. Dressings can be further classified into traditional, modern dressings and advanced dressings.

1.5.1. Traditional Dressings

These are the most common type of dressing material used for burn wound management (Balasubramani *et al*., 2001).Cotton wool, natural or synthetic bandages and gauze are traditional dressings which possess high absorption capacity and can be used as primary or secondary dressing. Due to their highly absorptive nature, wounds become dehydrated easily and may adhere to the wound, which in turn creates problems during dressing removal. Exudates or fluid leakage from the dressing may cause infection at the site. Most

traditional dressings are very cost effective when compared to other type of wound dressings.

1.5.2. Modern Dressings

It has been reported that wounds in a moist environment heal faster compared to wounds in a dry environment (winter, 1995). Modern dressings are classified based on the material they are made of and are grouped into categories such as hydrocolloids, alginates, foams and gels. Dressing materials which come under the category of modern dressings possess the ability to retain as well as create a moist environment around the wound to accelerate wound healing.

1.5.2.1. Hydrocolloids

Hydrocolloid dressings are combination of colloidal materials with other type of materials such as elastomers and adhesives. Classical hydrocolloids are composed of 40% of poly isobutylene, 20% of sodium carboxy methyl cellulose, 20% of gelatin, and 20% of pectin (Cockbill and Turner, 2007). They are used for light to moderately exuding wounds. The dressing absorbs the wound exudates and changes to a gel-like mass and provides a moist environment over the wound bed. The occlusive outer cover of hydrocolloids permits water vapor transmission between the wound and its surroundings. The high absorptive capacity of hydrocolloid dressings help to keep the wound clean. Changing the dressing before the gels leak out to avoid contamination is recommended. Some of the commercially available hydrocolloid dressings are Granuflex, Aquacel and Tegasorb, etc.

1.5.2. 2. Alginate Dressings

Alginate dressings are prepared from the sodium as well as calcium salts of alginic acids. The dressings can be used in the form of foams (freeze-dried porous sheets) or as flexible fibers. When alginate dressings come in contact with the wound, an ion exchange reaction takes place between the sodium ions of the exudates and the calcium ions of the dressing, and consequently, the fibers swell and dissolve to form a protective film of gel over the wound. The gelling process helps to maintain the moisture content and healing temperature. Alginate dressing promotes wound healing because the calcium ions present in the dressing can improve some cellular aspects of the wound healing process. They are therefore useful for moderate to highly exudative wounds (Thomas, 2000). The dressing cannot be used for non-exudative dry wounds. Some commercially available alginate dressings include Sorbsan, Curasorb and Kaltostat.

1.5.2.3. Foam Dressings

Foam dressings are made up of porous polyurethane foam mesh or polyurethane foam film. They are high absorptive in nature and maintain a moist environment around the wound. They possess water as well as gas permeability properties which help in re-epithelialization. Due to their high absorptive ability and moisture vapor permeability, foam dressings can be used as primary dressings (Ramos-E-Silva *et al*., 2002). Foam dressings cannot be used for dry epithelializing wounds. Examples of foam dressings include Lyofoam and Allevyn.

1.5.2.4. Hydrogel Dressings

Hydrogels are three-dimensional, hydrophilic polymer networks capable of imbibing a large amount of water. They are excellent materials and possess all the properties required for an ideal wound dressing material (Morgan, 1999). Hydrogels have the capacity to absorb wound exudates and safely retain them within the gel, which provides the microclimate that stimulates and regulates most of the cellular functions and nutritional processes during the individual phases of wound healing. This type of dressing can also be easily removed from the wound surface because of its non-adherent character (Thomas S *et al*., 1996). Due to its non-adherent property, hydrogel is comfortable to the patient and less painful when the dressing is being removed. The high water content of the hydrogel matrix provides a soothing as well as cooling effect to the wound site. The cooling sensation of the hydrogel dressing may cause reduction in pain (Ramos-E-Silva *et al*., 2002).

1.6. Polymeric Hydrogels

Hydrogels are hydrophilic polymer networks capable of imbibing large quantity of water (>20%). They find a wide variety of biomedical applications in drug delivery systems, tissue engineering and regenerative medicine, artificial organs, diagnostics and wound dressing. Hydrogel mimics properties (both physicochemical and mechanical) of the natural tissue in its swollen state. The swelling capacity of hydrogel is responsible for its high hemo-compatibility, structural integrity and elasticity. A number of natural and synthetic polymers, including chitosan, collagen, alginate, poly (n-vinyl pyrrolidone), poly vinyl alcohol and poly (2-hydroxy ethyl methacrylate, pHEMA) have been used for

the preparation of the hydrogel wound dressing (Azad *et al*.,2004). Various forms of hydrogels including gel-impregnated gauzes, sheets, plasters and amorphous gels are commercially available for the treatment of minor burns and other type of wounds. Hydrogel sheets and gel impregnated gauzes are also found to be suitable for the treatment of superficial burns.

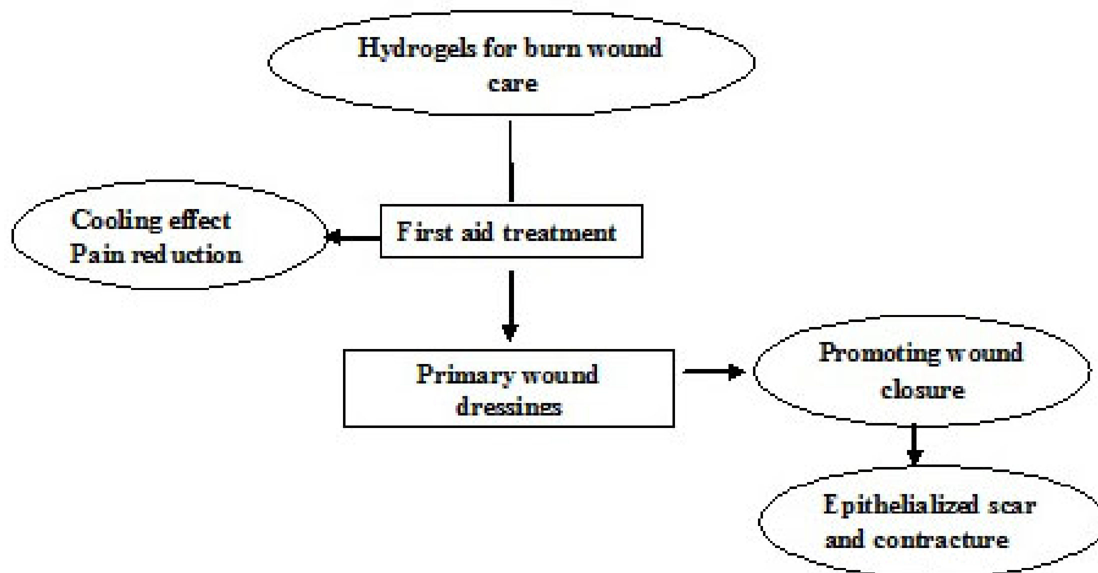

Figure 5. Role of hydrogel dressings in burn wound management

1.6.1. Poly (2-hydroxy ethyl methacrylate)

In 1955, Wichterle and Lim synthesized the revolutionary biomaterial called hydrogel from the monomer 2-hydroxy ethyl methacrylate (HEMA), which possesses potential biomedical applications such as contact lenses, intraocular lenses, drug delivery systems and wound dressings (Wichterle and Lim, 1960, Nierzwicki *et al*., 1975, Hoffman, 2006). pHEMA hydrogel possesses high biocompatibility, excellent blood compatibility and

good swelling characteristics. The hydrophilic nature of the polymer imparts a high degree of swelling to the hydrogel systems (Faxalv *et al.,* 2011, Singh DK, 1994). The main disadvantages of pHEMA hydrogels are their low mechanical strength and tear resistance. The problem of poor mechanical strength of these hydrogels can be resolved in many ways, especially by incorporating them with other polymer systems, adding cross-linking agents or copolymerization. Reports have shown that incorporation of poly caprolactone and poly ethylene glycol in the pHEMA hydrogel improves the psysico-mechanical property of hydrogel systems (Florence *et al*., 1994, Jung *et al*., 2006).

1.6.2. Poly (•-Caprolactone) PCL

This study selected PCL in order to impart mechanical properties to the polyHEMA hydrogel system. PCL is an FDA-approved polymer system which possesses excellent biocompatibility as well as good hemocompatibility. It is a semi-crystalline polymer with a glass transition temperature of -60^{0}c and a melting temperature of 55-65^{0}C. The repeating structure of PCL contains five non-polar methylene groups and a single hydrophilic ester group. Presence of the olefinic group provides structural properties similar to polyolefin while the hydrophilic ester group is responsible for the degradation property.

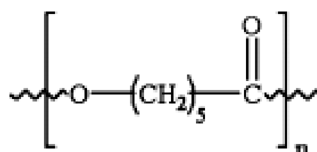

Figure 6. Representative image showing the structure of PCL

1.6.3. Poly Ethylene Glycol (PEG)

Poly ethylene glycol is a biocompatible polymer and is well known for its non-thrombogenic property. PEG is also used in the study to impart the plasticizing effect in the pHEMA-PCL system.

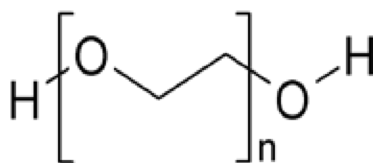

Figure 7. Representative image showing the structure of PEG

1.7. Antimicrobial Wound Dressing

Infection is the major concern of burn injury which accounts for the increased mortality and morbidity of burn patients. It is reported that the bacterial strains associated with burn wounds change with time (Merlin G *et al.*, 2008). Both gram-positive and gram-negative bacteria are responsible for the burn wound infection. Initially, gram-positive bacteria from the patient's cutaneous flora attack the wound and this is followed by the invasion of gram-negative bacteria from the patient's gastrointestinal, respiratory track and also from external contaminated surfaces. In practice, topical antimicrobial agents such as butadiene, silver sulphadiazene, etc., are applied to prevent and treat burn infections. At the same time, the wound have to be kept closed to prevent the invasion of microbes from the external environment. Topical application of the ointments has to be done once or twice a day which may cause discomfort to the patient. Therefore, the development of

wound dressings incorporated with antimicrobial agents to control infection at the wound site is more important. Delivery of antibiotic agents to the wound site locally may have some advantages over its systemic administration. Antibiotic overdose may result in cumulative cell and organ toxicity of the amino glycosides in the ears and kidneys. Application of wound dressings with controlled delivery of antibiotics can provide tissue compatibility and lower bacterial resistance and ensure little with wound healing.

Some of the reported wound dressings incorporated with antimicrobial agents include bilayer chitosan with controlled delivery of silver sulphadiazine, a combined form of cellulose acetate, PVA and gelatin for the delivery of nanosilver, chitosan-polyurethane film dressing with minocycline and pHEMA with ciprofloxacin (Mohdy *et al*., 2013, Aoyogi *et al*., 2007). Application of silicone gel sheets with controlled delivery of antibiotic ofloxacin to burn wounds have also been reported (Sawada *et al*., 1990).

1.7.1. Silver nanoparticle in wound dressing

Silver nanoparticles (SNPs) have long been known to possess excellent antimicrobial effects due to their high surface area to volume ratio and specific physico-chemical properties. SNPs modulate cytokine action in wound healing and also strongly inhibit biofilm formation at the wound site (Tian *et al.*, 2007). They have been used as an effective antimicrobial agent against gram-positive and gram-negative bacteria and also have anti-inflammatory effects which help healing of burn wounds (Pal *et al* ., 2007, Shahverdi *et al* 2007., Tian J *et al .,* 2007). Commercially available antimicrobial wound dressings with nanosilver particles as an antimicrobial agent are Anticoat, Silvercel, Aquacel Ag, etc. It is reported that, silver nanoparticles induce antimicrobial activity at

very small concentrations (Madhavan *et al*., 2011). The antimicrobial activity mechanism of SNPs can be explained in three ways as shown in Figure 8.

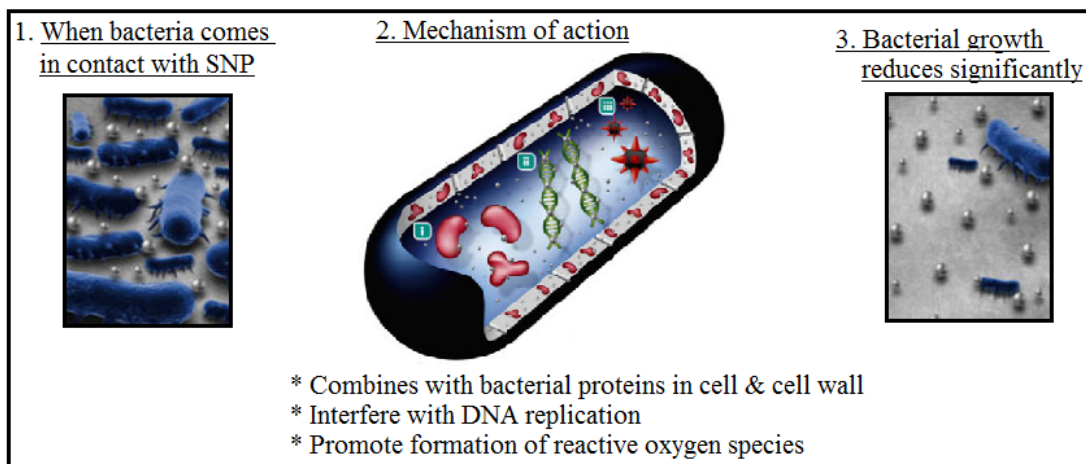

Figure 8. Schematic image showing mechanism of antibacterial action of SNPs

1.7.2. Fluoroquinolone antibiotic - Ofloxacin

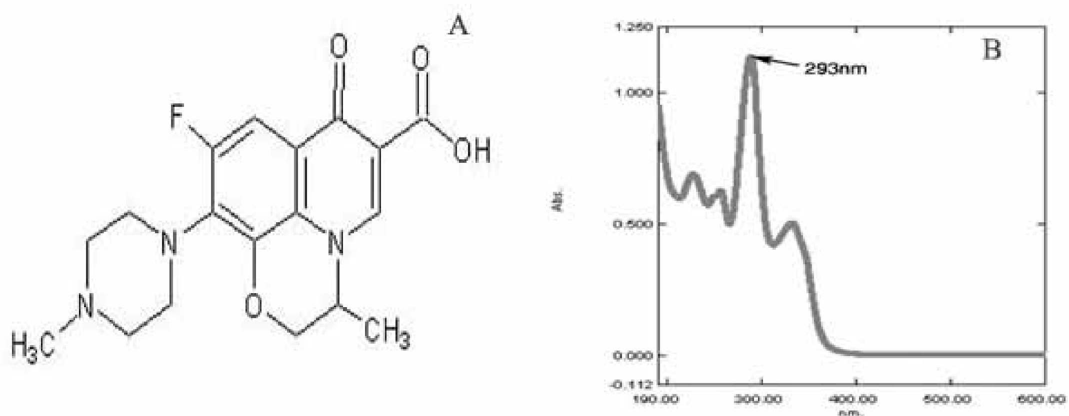

Figure 9. (A)Structure (B) UV spectrum of ofloxacin

Ofloxacin (Figure 9A, 9-Fluoro-2-3 dihydro(3- methyl)-10-(1-methyl piperazinyl)-7-oxo-7H-pyrido [1,2,3-de]- 1,4 benzoxazin-6-carboxylic acid) is a second generation fluoroquinolone (Al Omar, 2009). It possesses a broad spectrum of antibacterial activity against gram positive and gram negative bacteria and is found to be effective in controlling burn wound infection (Koushik *et al*., 2001). Ofloxacin inhibits the DNA replication by binding with enzymes DNA gyrase and topoisomerase IV, which may eventually lead to the death of the microbe. Figure 9B shows the UV spectrum of ofloxacin. Ofloxacin possesses specific spectral properties which facilitate the *in vitro* evaluation of drug release. An aqueous solution of Ofloxacin gives maximum absorption at 290 nm which corresponds to the carboxylic chromophore involving the nitrogen of the piperazinyl to the carbonyl group.

1.8. Wound Dressing Incorporated With Growth Factors

Growth factors play a prominent part in the wound healing process and actively take part in cell division, migration, protein expression and enzyme production, which are essential for proper wound healing. These polypeptides are secreted by the cells at the wound site and can effectively participate in the wound healing process by stimulating angiogenesis and cellular proliferation. Growth factors play a major role in the inflammatory phase, fibroblast activity, and synthesis and decomposition of the extracellular matrix (Komarcevic, 2000). The growth factors which are involved in wound healing are epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and transforming growth factor (TGF-• 1). The epidermal growth factor secreted by keratinocytes at the wound site helps in re-epithelialization. Both *in–vitro* and

in-vivo studies have shown that EGF stimulates the growth of keratinocytes and helps in wound healing (Ulubayram *et al*, 2001). Topical application of EGF cannot provide significant improvement in wound healing because of the very short half-life of EGF. Many proteases present in the burned site may decompose and deactivate the biological function of EGF at the site when it is applied as an ointment (Okumura *et al.*, 1990). It has been reported that incorporation of EGF into a polymer matrix and their sustained release to the site can enhance the *in-vivo* efficiency of EGF. Natural polymers such as gelatin, alginate, dextran, collagen and synthetic polymers including polyacrylamide and polyurethane films have been studied as carrier systems for the sustained release of EGF to the wound site (Ulubayram *et al*, 2001))

1.9 Photo-polymerization method

Hydrogels can be prepared using different polymerization techniques. Conventional techniques include redox initiation, ionic initiation and addition mechanisms. Photo-polymerization, on the other hand, has several advantages over conventional techniques. It has spatial as well as temporal control over polymerization, faster rate (less than a second to few minutes) at room or physiological or even higher temperatures, and minimum heat generation (Decker et *al* 1980). Photo-polymerization is usually achieved using organic photo-initiators which can be UV or visible-light active. Commonly used photo-initiators are 2, 4, 6 trimethyl benzoyl biphenyl phosphine oxide (TPO), campharoquinon (CQ), etc. In the present work, we have used TPO as a photo initiator.

1.9.1. Rationale for choosing 2, 4, 6 trimethyl benzoyl biphenyl phosphine oxide (TPO)

The major problem associated with the commonly used photo-initiators such as camphorquinone is that they tend to impart color to the polymer system. In our study, we have used a novel photo-initiator.2, 4, 6 trimethyl benzoyl biphenyl phosphine oxide (TPO) which facilitates free radical polymerization at 410 nm.

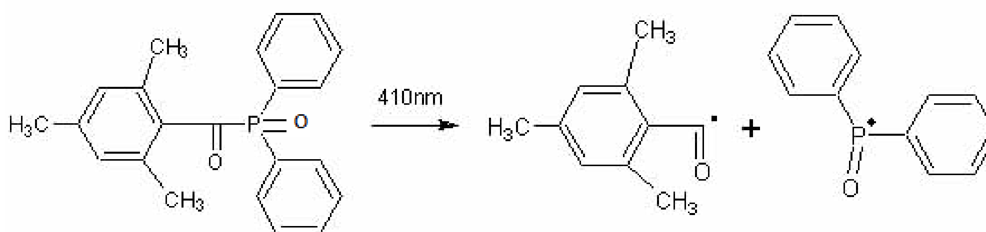

Figure10. Photo-cleavage of TPO at 410nm

Advantages of TPO

- Polymerization can be done at room temperature or physiological temperature
- Non-cytotoxicity
- Does not impart any color to the system
- Faster rate of polymerization

2.0. Limitations of available burn wound dressings

There are large number of wound dressings are available in the market. Most of them are too expensive for the common man to afford. One of the important properties of an ideal dressing is patient's comfortability .The tendency of the wound dressing to adhere to the

wound site causes increased pain and discomfort to the person on removal of the same. Most of the dressings based on hydrogel have a capacity to hold large amount of fluid within them, which may lead to skin maceration and bacterial proliferation which produces a foul smell in infected wounds (Boateng *et al*., 2007). Moreover, low mechanical strength of hydrogel may cause difficulty in proper handling and may affect patient compliance (Martin *et al*., 2002). In the case of antimicrobial wound dressings, the uncontrolled release of antimicrobial agents may cause toxicity and delayed wound healing (McCauley, 1989, Lineweaver, 1985). Some of the wound dressing which contain silver as antimicrobial agent may cause damage to normal tissue. Furthermore, some of the medicated dressings with antibiotics may produce antibiotic resistance at the site. Major problem associated with growth factor incorporated wound dressing is the deactivation of growth factor within the polymeric matrix during the formulation procedure. The major difficulty in developing ideal burn wound dressing is the broad diversity of burn wounds, differing in-depth, size and location. Nature of healing may be affected by factors such as infection, patient's age, nutritional status, associated injuries etc.

2.1. Hypothesis

Most burn injuries may lead to either mortality or morbidity. Proper wound management is necessary to resolve the complications associated with the burns. Prevention of bacterial invasion from the environment as well as from the patient's own body may improve the quality of burn wound management. Incorporation of antimicrobial agents in the wound dressing may facilitate bactericidal effects on the dressing. Though

antimicrobial agents can participate in controlling infection at the wound site, the promotion of wound healing can be achieved by the incorporation of growth factors in the wound dressing

(1) Development of a non-adherent and cytocompatible hydrogel matrix based on pHEMA as a wound dressing material using a unique photo-polymerization technique

(2) Development of an antimicrobial hydrogel matrix to control the infection at the wound site using multiple approaches

(3) Development of a growth factor incorporated hydrogel matrix to promote wound healing

2.2. Objectives of the study

1. To prepare a poly(2-hydroxy ethyl methacrylate)-poly(caprolactone)-poly(ethylene glycol) [pHEMA-PCL-PEG] hydrogel matrix by the photo-polymerization technique using the photo-initiator 2,4,6 trimethyl benzoyl biphenyl phosphine oxide (TPO) and their optimization
2. To characterize the physico-mechanical properties of the pHEMA-PCL-PEG hydrogel matrix as a wound dressing material
3. To fabricate and characterize antimicrobial hydrogel wound dressings using silver nano particles (SNPs) and ofloxacin antibiotic
4. To evaluate the elution profile of antimicrobial agents and their antimicrobial property
5. To develop a hydrogel matrix incorporated with EGF (H-EGF) and its characterization

6. To evaluate the surface morphology and hydrophilic character of H-EGF
7. To analyze the elution profile of the epidermal growth factor from the matrix H-EGF and to study the effect of the growth factor on fibroblast growth

2.3. Burn Wound Dressings- Market Scenario

Indian market for wound dressings constitute collagen based dressing (32%), hydrogel based dressing (22%), active dressing such as skin replacement (9%), foam dressing (17%), film dressing(10%) and other types of dressing(4%). Major companies involved in manufacturing these dressings are 3M Co, Smith and Nephew and Johnson & Johnson. Reports on global and national status of burn care products show that global market of wound care products is expected to reach USD 2.33 billion by 2020 while Indian market is expected to reach INR 22 billion by 2019.

CHAPTER 2

LITERATURE REVIEW

The major objectives of the study are to develop hydrogel-based polymer systems intended for burn wound dressing applications in order (1) to provide a covering function (2) to control infection at the wound site, and (3) to provide the wound site with a growth factor that would promote wound healing. Given these goals, it is necessary to evaluate the current progress in this field.

2.1. Wounds and wound healing

A wound, which is defined as the disruption of the normal anatomic structure and function of skin, can be caused in different ways such as mechanical as well as physical damage, and thermal injury. According to an American Burn Association report (American Burn Association Report., 2005) thermal injury is the major cause of significant skin loss. Infection and fluid loss are lethal problems associated with burn wounds.

A wound can be described as a break or defect in the structure and function of skin caused by physical or thermal agents. Burn wound are a kind of wound which occurs when the skin is damaged by heat, electric current, chemical or flammable objects. Burn wounds are classified into four groups according to the depth and the affected layers, namely, superficial burns, partial thickness burns, full thickness burns and fourth degree burns. In superficial burns, only the epidermal layer is affected. The burn area is

characterized by edema which diminishes after 24 h. The wound generally heals within a week (Whitney and Whickline, 2003). Partial thickness burns are deeper than superficial burns as they affect the epidermis as well as the dermis, and require extensive time for healing, i.e., nearly 3-4 weeks. Full thickness burns affect all three layers of the skin. Skin appendages such as sweat glands and hair follicles are also destroyed. Fourth degree burns extend through the skin layers into the underlying ligaments and muscles.

2.2. Principles of wound healing

Wound healing is a complex biological process which relates to physiological parameters. Knowledge of the wound healing process is necessary for the selection of suitable wound dressing material. Various biological and physiological processes are involved in the wound healing process. These can be summarized into five overlapping stages – hemostais **,** inflammation, migration, proliferation and remodeling.

The first stage of wound healing involves hemostasis which occurs soon after the injury. In this stage, blood vessels at the injury site constrict to control the bleeding. Fibrinogen in the wound exudates leads the clotting process, resulting in coagulation of the exudates and together with the formation of the fibrin network, produces a clot. Scab, the dried form of a clot, provides strength and support to the injured tissue. The inflammatory phase is the second phase of wound healing, which occurs almost simultaneously with the hemostatic phase. This phase involves vascular as well as cellular responses. Vascular response is characterized by vasodilation . In this stage, permeability of blood vessels increases which makes easy infiltration of monocytes and

neutrophils to the injury site. The neutrophils scavenge the cellular debri, bacteria and foreign bodies at the wound. Macrophages, differentiated from monocytes, help in the phagocytosis of the cellular debri and bacteria and secrete growth factors. The growth factors activate and attract fibroblasts, local endothelial cells and keratinocytes to the wound site and initiate the next stage of wound healing.

2.3. Wound Management

The ideal wound management is one that provides effective healing of the wound within minimal time and with optimal comfort to the patient. Effective wound management can be achieved by using an ideal wound dressing material. An ideal dressing material should: absorb exudates from the wound and simultaneously provide a moist environment, not produce any toxic effect, be non-adherent to the wound, and restrict the entry of pathogens to keep the wound clean. The physiological conditions of the wound would also influence the properties of the wound dressing.

2.4. History of wound dressings

A range of methods has been used to treat wounds, dating back to ancient times. In early times, natural materials were used to protect wounds from the external environment. The earliest known record of the treatment of wounds was found on clay tablets of Mesopotamian origin from about 2500 BCE (Singer *et al.*, 1998). In ancient times, wounds were cleansed with water or milk and then covered with honey or resin (Forest, 1982). The management of wounds had its beginnings in ancient Egypt using grease-soaked gauze bandages. Prior to the 1960s, dry environment was considered as an

optimum condition for wound healing (Cho *et al*., 1998). But clinically, dry environment does not favor wound healing and also shows adherence to the wound. In 1962, Winter demonstrated that a moist environment is essential for rapid re-epithelialization and wound closure rather than a dry environment (Winter, 19962). In 1985, Turner described the performance parameters of an ideal wound dressing which would help the dressing to provide a suitable microclimate and facilitate proper wound healing (Turner, 1985). According to Turner, a dressing material should

- Remove excess exudates and toxic components
- Maintain a high level of humidity at the wound/dressing interface
- Allow gaseous exchange
- Provide thermal insulation
- Protect against secondary infection
- Be nontoxic
- Not produce trauma/pain on removal

A moist environment has been proven to be highly effective in wound healing as it facilitates fibroblast proliferation, keratinocytes migration, collagen synthesis, and angiogenesis (Balton *et al*., 2000).

2.4.1. Wound dressings –Modern Era

The most common, inexpensive and readily available wound dressing material is plain gauze made up of cotton wool and bandages. The term gauze represents two types of bandaging material (a) woven gauze – 100% natural cotton cloth and (2) non-woven gauze – synthetic dressing made of a blend of rayon or synthetic fiber (Jones, 2006).

These dressing materials performed their primary function of wound protection and prevention of bleeding (Sheridan *et al*., 1999).The quality of cotton-based wound dressing subsequently improved by using active ingredients such as iodine, zinc oxide/zinc ions or petrolatum. Addition of iodine provided the antibacterial property while zinc oxide facilitated wound cleansing and re-epithelialization (Murphy *et al*., 2012, Agren, 1990). Gauze dressings dipped in antibiotics such as EUSOL, chlorhexidine or proflavin were used to control infection and promote granulation at the wound site. Nevertheless, the use of gauze often results in problems associated with its removal as it may cause trauma and associated pain by stripping off the newly formed epidermis (Stashak *et al*., 2004). Moreover, one of the biggest problems associated with the woven gauze is the foreign body reaction caused by cotton fibers. The greatest advantage of gauze-based materials is their low cost.

Dressing material	**Brand Name**	**Manufacturer**
Parrafin gauze dressing with chlorohexidine acetate (5%)	Bactigras	Smith & Nephew
Parrafin gauze dressing	Jelonet	Smith & Nephew
Scarlet Red dressing	Scarlet Red	Chesebrough-Pond's Inc.
Petrolatum gauze	Xeroform	Chesebrough-Pond's Inc.
Highly absorbent cotton wool pad	Gamgee pad	3M
Absorbent cotton pad	Telfa ‘‘ Ouchless’’ non adherent dressings	Kendall(Covidien)
Rayon/cellulose blend sandwiched with a layer of anti-shear high density poly ethylene	Exu Dry Dressing	Smith & Nephew

Table 1. Commercially available gauze-based wound dressings

2.4.2. Advancements in the 20th century

2.4.2.1. Moist wound healing

Advanced wound dressings have been developed mainly focusing on the 'moist wound healing' aspect. Though Winter demonstrated the importance of moist wound healing way back in 1962, it received serious attention only recently (Winter, 1962). Dressing designed for moist wound healing is represented by hydrogel and hydrocolloid products. Both induce autolytic debridement which helps to keep the wound clean.

Hydrocolloids are usually composed of pectin, sodium carboxy methyl cellulose, gelatin, elastomers and adhesives. Hydrofiber is a commercially available hydrocolloid dressing in the form of hydrophilic non-woven flat sheet dressing. Upon moisture absorption, the dressing is converted into a swollen gel mass and forms a protective layer to cover the wound. Some of the commercially available hydrocolloid dressings are Aquacel and Granuflex ™ (ConvaTec, UK), Tegasorb ™ (3M Healthcare, UK) and Comfeel ™ (Coplast, UK) (Boateng *et al*., 2015). They can be used for the management of minimal to moderately exudating wounds but are not suitable for dry wounds.

Hydrogels are insoluble hydrophilic polymer networks made from natural as well as synthetic polymers. The highly water absorptive capacity of hydrogel makes it a suitable candidate for wound management. It can be applied to dry wounds since it does not need wound fluid to become a gel (Murphy *et al*., 2012) as well as to exudating wounds.

2.5. Hydrogels –Polymers

The term hydrogel refers to a broad class of polymeric materials which have the capacity to absorb fluid in greater amount. It can be prepared from natural as well as synthetic polymers. After Wichterle and Lim synthesized pHEMA hydrogels in 1955, researchers modified hydrogel systems for various applications. Yannas *et al* modified (Yannas and Bruker, 1980) synthetic hydrogels with some natural substances such as collagen and shark cartilage to obtain novel burn wound dressings. Shah et al. reported that a composite of cotton gauze impregnated with a thermoplastic hydrogel was suitable for wound management (Shah *et a*l., US Patent No: 5,527,271).

The process of hydrogel formation is essentially due to monomer polymerization or cross-linking of preformed polymers. A large number of methods have been employed to perform hydrogel formation including physical as well as chemical methods. Of these, photo-polymerization has been reported as an effective and simple method of hydrogel preparation by monomer polymerization. Rosiak *et al.* reported the methodology of gamma radiation to obtain sterile hydrogel for wound management (Rosiak et al., 1993, Rosiak, 1991). They used both synthetic polymers such as polyvinyl alcohol and poly vinylpyrrolidone and natural polymers such as agar and gelatin. Lopergolo *et al* reported PVP hydrogel preparation using high energy radiation (Lopergolo *et al*., 2002). Synthesis of pHEMA hydrogel systems was effectively accomplished using the UV radiation technique (Young et al., 1998).

2.5.1. Hydrogel wound dressings - superficial and partial thickness burns

The intrinsic potential of hydrogels to promote skin healing has been increasingly investigated and applied in clinical settings in the early eighties. Hydrogels were found to absorb and retain the wound exudates, thus promoting fibroblast proliferation and keratinocytes migration which are necessary for complete epithelialization of the wound (Winter., 1962, Bullock *et al.,* 2010). In addition, the tight mesh size of hydrogels (in the order of 100 nm in the swollen state) prevents bacteria from attacking the wound (Drury *et al*., 2003). Wearing comfort and immediate pain relief are the most beneficial features of hydrogels compared to other types of dressings. In the case of burn wounds, application of hydrogel provides a cooling sensation to the wound, which minimizes the extent of damage and also alleviates the pain. The cooling sensation as well as the soothing effect can be attributed to its high water content.

As far as burn wound dressing is concerned, the non-adhesive nature of hydrogel is another important property. Because of its hydrophilic property as well as the low interfacial tension between the hydrogel surface and the surrounding solution, cells do not adhere to the dressing surface. This property makes the removal of dressing easy, without causing pain and bleeding, and improves patient comfort. Another important parameter is the transparency of hydrogel systems which helps monitoring the wound healing stages without removing the dressing. A large number of hydrogel systems are commercially available for the treatment of burns and other wounds in different forms such as amorphous gels, gel impregnated gauzes and sheets. Sheets and gel impregnated gauzes find application in the treatment of superficial wounds (Winter, 1962). In spite of the

availability of various hydrogel-based wound dressings in the market, the development of advanced hydrogel dressings still represents a very active field of research with the aim of further improving skin healing in relation to specific wound healing aspects. The following are some of the commercially available hydrogel wound dressings:

Product	Manufacturer	Constituents
Granugel	ConvaTec	Pectin, carboxymethyl cellulose and propylene glycol
Intrasite Gel	Smith & Nephew	Modified carboxy methylcellulose (2.3%) & propylene glycol (20%)
Purilon Gel	Coplast	Sodium carboxymethyl cellulose and water (>90%)
Aquaflo	Covidien	Polyethylene glycol and propylene glycol
Woundtab	First Water	Sulphonated copolymer, carboxymethyl cellulose, glycerol and water

Table 2. Commercially available hydrogel based wound dressings

2.6. pHEMA hydrogels –Wound dressing

Poly (2-hydroxy ethyl methacrylate) based hydrogel is one of the most important biomaterials currently available. After pHEMA was developed, it attained greater importance as wound dressing material over a period of time due to its superior properties. Advantages of pHEMA systems include swelling capacity, inertness to biological processes, resistance to degradation and absorption by the body. Various researchers have studied the use of pHEMA hydrogel as a wound dressing material (Siriwittayakon *et al*., 2001, Prasitslip *et al*., 2003). The presence of the hydroxyl and carboxyl groups makes the polymer compatible with the water while the hydrophobic entity methyl group and back bone provide hydrolytic stability and mechanical strength to

the polymer. The copolymer system of pHEMA and PEG-MA hydrogel systems exhibited resistance towards non-specific protein adsorption and cell adhesion (Kwon *et al.*, 2003). pHEMA hydrogel film was used as a control in the study of the effect of synthetic dressing in burn wound infections (Nathan *et al.*, 1974). Furthermore, the free hydroxyl group of the polymer system can be used to facilitate immobilization of other molecules such as collagen, which have a greater role in wound healing (Martin *et al.,* 2003).

2.7. Fluid absorbing capacity of hydrogel systems

The fluid uptake ability of hydrogel is the most important property that makes it suitable for wound management. The fluid absorbing capacity of hydrogel makes it similar to natural living tissue (Calo *et al*., 2014). It provides a cooling sensation which may cause reduction in pain and enhance patient acceptability. Moody et al reported that hydrogel wound dressing could successfully reduce the pain in the case of chronic leg ulcer (Moody et al., 2006). The high water content of hydrogel helps promote moist wound healing. The high water content of hydrogel is accountable for the increase in the biocompatibility of hydrogel systems (Davis *et al.,* 1991) and makes it suitable for use at all four stages of the wound healing process (Morgan, 2002).

2.8 Medicated dressing for delivery of therapeutic agents

A relatively new approach to wound healing involves the use of polymer dressing to deliver various pharmacological agents such as antimicrobials/antibiotics, growth factors, anti-inflammatory agents, etc., which play an active role in wound healing directly or indirectly. Traditional dressing materials such as plain gauze and paraffin impregnated

gauze and modern dressings include hydrogels, hydrocolloids and polyurethane film/foams, which are found to effectively participate in drug delivery at the wound site.

2.8.1. Wound dressings with silver as an antimicrobial agent

The antimicrobial property of silver has been known for many centuries and silver has a long history of use as a clinical antimicrobial agent. During the 17th century, silver nitrate solution was used against postpartum eye infection in newborns (Silvestry *et al*., 2007). Diluted silver nitrate solution (0.5%) was first used in burn wound care in 1964 and this attracted research interest in silver nitrate subsequently (Moyer *et al*., 1965). The breakthrough discovery of silver sulfadiazine cream for the treatment of burn wound infection in the late 1960s prompted the use of silver in topical antimicrobial agents over the last four decades (Fox, 1968, Fox *et al*., 1974).The progress of nanotechnology also resulted in the development of silver nanoparticles, which control infection and is cytocompatible with human cells (Rizello *et al*., 2014). *In vivo* studies of SNP-incorporated wound dressings were found to exhibit excellent inhibitory action against *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Ong *et al*., 2008). Mohdy et al reported that silver nanoparticle-impregnated wound dressing based on cellulose acetate/PVA/gelatin hydrogel exhibited strong inhibition against infection and simultaneous wound healing (Mohdy *et al* .,2013).An antimicrobial organic-inorganic hybrid hydrogel with controlled release of silver ion showed excellent antimicrobial activity .The antibacterial effect of SNP-incorporated PVA-PVP hydrogel wound dressings was well studied and found to be excellent for controlling infection *in vitro* (Yu *et al*., 2006). 2-acrylamido-2-methylpropane sulphonic acid sodium salt hydrogel-

incorporated with SNPs was found highly effective in controlling burn wound infection *in vivo* (Benjawan *et al*., 2014).

2.8.2. Wound dressings with antibiotics

As far as a burn wound is concerned, the major concern is infection, which could lead to morbidity as well as mortality. Controlled delivery of drugs to the wound site using a wound dressing in a controlled consistent and sustained fashion over a long period of time, without the frequent change of wound dressing .The delivery of antibiotics using wound dressing has certain advantages such as (1) just a small quantity of drug is needed to achieve the desired properties to control infection at the wound site, which reduces the risk of systemic toxicity (2) the use of dressing may provide tissue compatibility, low occurrence of bacterial resistance and reduced interference with wound healing (Chuo *et al*., 2006, Doillon *et al*., 1986). Some of the reported hydrogel wound dressing systems incorporated with antibiotics such as poly (vinyl pyrrolidone), poly (vinyl alcohol) and poly (hydroxyl alkyl methacrylate) systems have proven their ability to function as a suitable dressing material to control infection (Hoffman et al., 2002, Kietzman et al.,2006, Thorn et al., 2006). Sawada et al reported that an ofloxacin-incorporated silicone sheet was effective in controlling burn wound infections *in vivo* (Sawada *et al*., 1990). Poly (2-hydroxy ethyl methacrylate) hydrogel systems incorporated with ciprofloxacin exhibited a broad spectrum of antimicrobial activity with controlled drug release (Tsou et al., 2005). Table 3 depicts some of the reported wound dressing materials incorporated with antibiotics.

Delivery systems	Drug	Group
PVA/Dextran	Gentamicin	Hwang *et al*,2010
Pullulan Hydrogel	Gentamicin	Li *et al*, 2011
PVA/PVP/Chitosan hydrogels	Ciprofloxacin	Yu *et al*,2005
Poly(N-isopropyl acrylamide)/ chitosan hydrogel	Ciprofloxacin	Radhakumary *et al*, 2011
Chitosan /Polyurethane	Minocyclin	Aoyogi *et al*,2007
Chitosan /PCL	Ofloxacin	Sahoo *et al* ,2010
Chitosan-PAAm	EGF	Pulat *et al* ,2013

Table 3. Wound dressings incorporated with antibiotics

2.8.3. Wound dressings with growth factor

Growth factors are a class of bio-macromolecules which are actively involved in the wound healing process. Reports have shown that epidermal growth factor (EGF) accelerated cellular proliferation and extracellular matrix synthesis. *In vivo* studies have proven that EGF is effective in epithelialization of human and animal wounds (Brown *et al*., 1989). Reports showed that various approaches such as topical application in the form of ointments, injections and use of controlled delivery systems, etc. have been used for EGF delivery to the wound site (Ulubayram *et al* .,2001). Of the various methods, encapsulation of EGF on the matrix is preferable because the short half life of the growth factor may adversely affect its properties. Buckley *et al* reported that sustained release of EGF from a subcutaneous pellet is highly effective in wound healing *in vivo* and superior

to daily injection of EGF (Buckley *et al*., 1985). Some of the reported wound dressing materials incorporated with growth factor are shown in table 4.Polymeric wound dressings were successfully developed for the incorporation of free growth factors using natural as well as synthetic polymers (Wang *et al*., 2015, Pulat *et al*., 2012). The solvent sorption method has been found to be an effective one to the load growth factor onto the matrix without affecting its activity (Dogan *et al*., 2004).

Wound dressing	**Growth Factor**	**Group**
Hydrogel dressing	TGF-•	Poulokkainen. *et al,*1995
Collagen film	PDGF	Koempal *et al,*1998
Polyurethane	EGF	Grzybowski *et al,*1999
Alginate	EGF	Koelwel *et al,*2008
Gelatin	EGF	Ulubyram *et al,*2004
Dextran	EGF	Dogan *et al,*2012
Chitosan-PAAm	EGF	Pulat *et al* ,2013

Table 4. Wound dressings incorporated with growth factors

2.9 Mechanism of drug delivery from hydrogel matrix

The unique physical properties of hydrogel such as high water content, softness, permeability to small molecules, low interfacial tension, etc., make it a suitable carrier for drug delivery application (Vashist *et al*., 2014). The advantages that hydrogel systems offer for drug delivery application include the possibility of sustained release of the drug to the site of application for a longer time period (Hoare *et al*., 2008). Reports have shown that hydrogel drug loading and drug release take place through different

mechanisms such as diffusion-controlled and swelling-controlled mechanisms (Calo et al., 2015).

2.9.1 Diffusion-controlled drug release

Drug release from a reservoir device or a matrix can be achieved through a diffusion-controlled mechanism. In this method, the drug is found to be released by diffusion through the hydrogel network or pores filled with water. The capsule represents the reservoir system in which the drug-containing core is coated with a hydrogel membrane. The concentration gradient between the core and membrane allows the release of the drug through diffusion (Figure 11). In a hydrogel matrix system, the drug is uniformly dispersed or dissolved throughout the matrix. In this case, drug release may be accomplished through a macromolecular network or pores and the release rate is proportional to the square root of time (Figure 12).

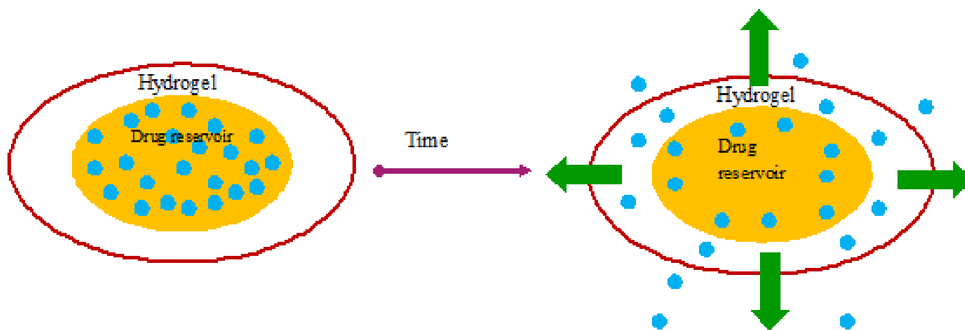

Figure 11. Drug release from a reservoir system by diffusion through entire hydrogel membrane

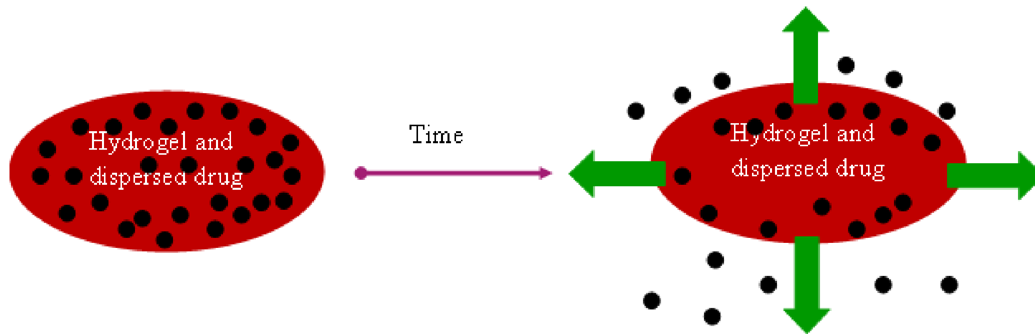

Figure 12. Drug release from a matrix system by diffusion through entire hydrogel system

2.9.2 Swelling-controlled mechanism

In a swelling-controlled release system, the drug is dispersed in a glassy polymer matrix and the drug release materializes through its swelling properties. When the polymer matrix comes into contact with water or bio fluid, it instigates swelling of the polymer. In the swollen stage, the glass transition temperature of the polymer is lowered, with the relaxation of polymer chains, which helps in the diffusion of the drug. Known as case II transport, this process is characterized by constant time-dependent release kinetics. In certain cases, the swelling controlled mechanism is found to be combined with diffusion, which is called anomalous transport (Bierbrauer, 2005, Pepppas *et al*., 1999).

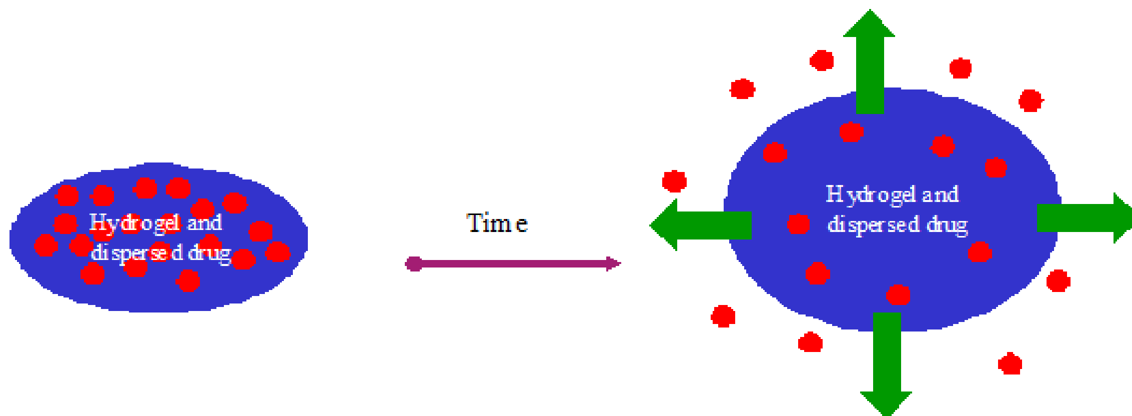

Figure 13. Drug release through swelling controlled release

2.10. Limitations of the current systems

World widely, development of wound dressing forms an important segment of the medical and pharmaceutical market. Various reports have shown that most of the available dressing possessed certain limitations such as high cost, low mechanical strength and adherence to the wound site (Margaret *et al*., 2007). Thomas *et al* reported that many of the medicated dressings in which antimicrobial agents are incorporated in the adhesive mass or paraffin base exhibited a problem of antimicrobial agent leakage in to the wound exudates (Thomas *et al*., 1990). Monafo showed that prolonged application of wound dressing with antimicrobial agent mafenide acetate may induce systemic toxicity which results in delayed wound healing (Monafo, 1992).

To summarize, it is clear that significant progress has been made in the development of wound dressing for proper wound management. It is essential to select the appropriate wound dressing to suit the particular type of wound for optimum healing and patient wellbeing. The development of an ideal wound dressing material with

adequate properties still remains a challenge. The present work focuses on showing that hydrogel wound dressings with adequate properties can serve the purpose and efforts are being taken to achieve this goal.

CHAPTER 3

MATERIALS AND METHODS

In this study, attempts were made (1) to develop novel hydrogel-based wound dressing materials with adequate strength which can provide temporary burn wound coverage, (2) to prepare a matrix which can deliver antimicrobial / antibiotic agent to the wound site to control infection at the site, and (3) to prepare a burn wound-dressing matrix which can release growth factor to improve wound healing effectively.

All hydrogel samples were prepared via a photo-polymerization technique using TPO, a unique photo-initiator. An antimicrobial hydrogel matrix was prepared incorporating PEG-protected silver nano particles (SNPs) and the antibiotic ofloxacin. Growth factor incorporation was accomplished using the solvent sorption method. All experiments related to hydrogel fabrication are described in section 3.1. The physico-mechanical property evaluation is detailed in section 3.2. Biological evaluation of the developed matrices is described in section 3.3.

3.1. Development of pHEMA-PCL-PEG hydrogel matrix

3.1.1. Commercial reagents

2-hydroxyethylmethacrylate [HEMA], 2, 4, 6 Trimethyl Benzoyl Biphenyl Phosphine oxide (TPO),Poly (ε-caprolactone) (PCL, Mw 14,000) and Ofloxacin (R&D grade) were all purchased from Sigma-Aldrich Chemical Company Inc., USA. Polyethylene glycol

(PEG, MW 200 and MW 3400) and silver nitrate (AR grade) were procured from Merck, Germany. Human recombinant epidermal growth factor (EGF) was obtained from Invitrogen, USA.

3.1.2. Fabrication of the hydrogel matrix using the photo-polymerization method

The HEMA monomer was distilled in vacuo before use to remove the inhibitor. PCL and PEG were added to the monomer and heated up to 70-75 ^{o}C to obtain a homogenous mix. The TPO photo-initiator (1wt %) was added to this mix under constant stirring. The solution was then poured into a Teflon mold (60 mm x 10 mm x 1 mm) and exposed to a dental light source containing a halogen bulb (Model 301, Prolite, Caulk/Dentsply, Melfork, DE, USA) having a wavelength 390-410 nm to initiate the polymerization. Light exposure was provided for a maximum of 2-3 minutes. After completing the polymerization, the mold containing the cured polymer was kept in distilled water till the polymer peeled away. Six hydrogel systems were prepared accordingly based on the formulations listed in Table 5. The hydrogel sheets were stored in distilled water at 37 ^{o}C.

Sample Code	Wt %		
	HEMA	PCL (14,000)	PEG(200)
HI-1	95	2.5	2.5
HI-2	90	5	5
HI-3	85	10	5
HI-4	80	10	10
HI-5	95	5	0
HI-6	90	10	0

Table 5. Composition of different hydrogel systems

The steps involved in hydrogel fabrication, viz., mixing of components, casting on the teflon mold and light curing using the light source prolite are presented in figure 14 and figure 15.

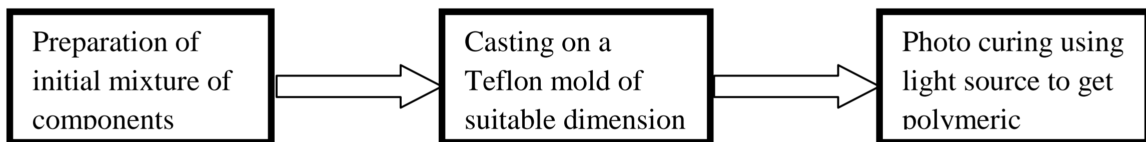

Figure 14. Schematic representation of steps involved in hydrogel preparation

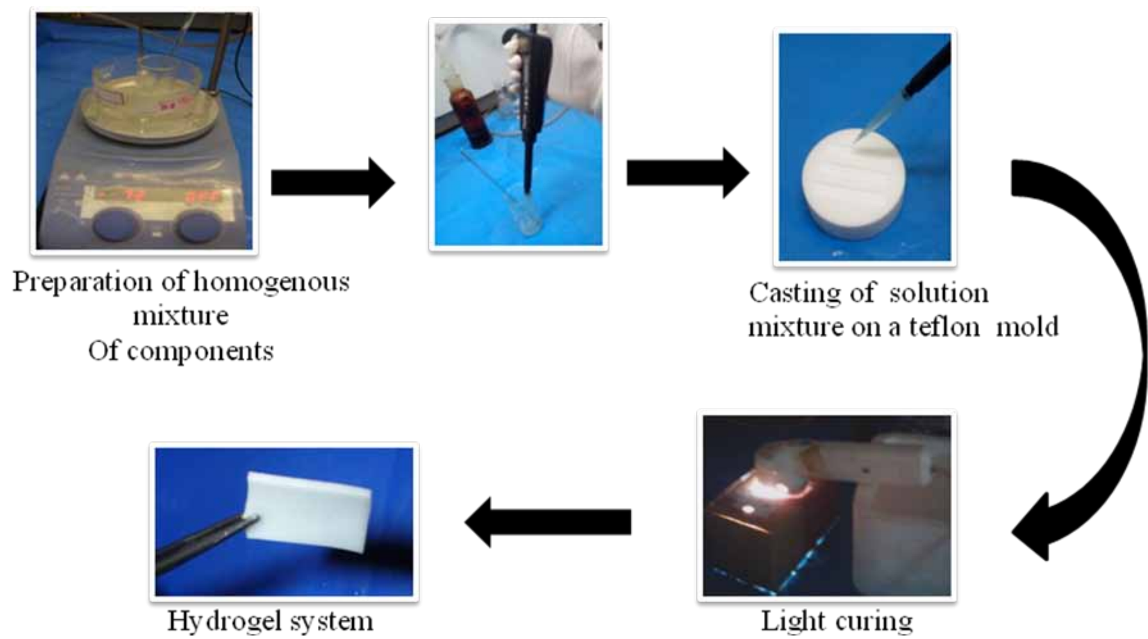

Figure 15. Fabrication of Hydrogel System

3.1.3. Synthesis and characterization of silver nano particles

The Polyethylene glycol protected SNP solution was prepared as shown in figure 16. About 3.88 g of PEG 3400 was dissolved in PEG200 (10 ml) at 80°C with stirring. After the complete dissolution of PEG, about 0.1 g of powdered silver nitrate was added stepwise to the PEG solution at 42-43 ^{o}C under constant stirring for 1 h. A clear brown color indicated the formation of SNP. Prepared SNP was characterized using transmission electron microscopy (TEM, Hitachi H-7650, Japan) and UV–visible spectroscopy (UV-1800, Shimadzu).

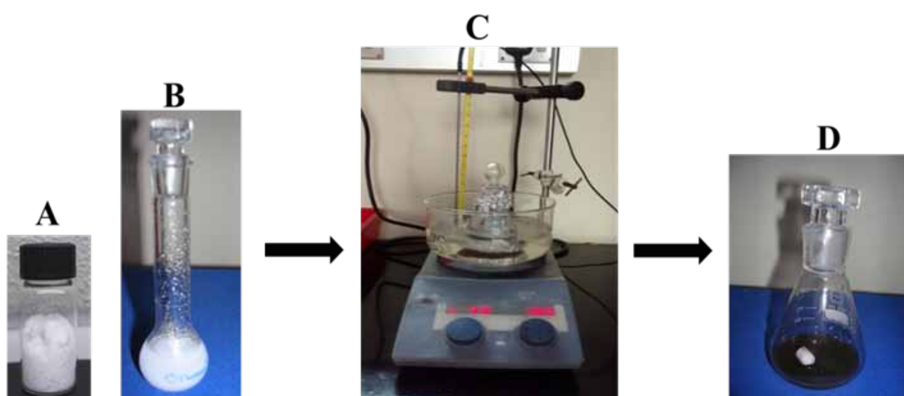

Figure 16.Procedure of SNP preparation A. Silver nitrate B. PEG solution prepared by dissolving PEG 3400 in PEG 200 C. Synthesis set up of SNP solution D. Prepared SNP solution

3.1.4. Fabrication of SNP-incorporated hydrogel matrix

Fabrication of the SNP-incorporated hydrogel system was carried out using photo polymerization as explained in section 3.1.2. The initial fabrication was carried out by adding the SNP solution into the HEMA-PCL-PEG mixture (85:10:5) containing 1.0 wt% of the photo initiator TPO in such a way that the composition contained silver nano particles amounting to 0.1wt% of the total polymer content. In second step, the

homogenous solution was transferred to the Teflon mold and this was followed by light curing to achieve the polymerization as depicted in Figure 17.

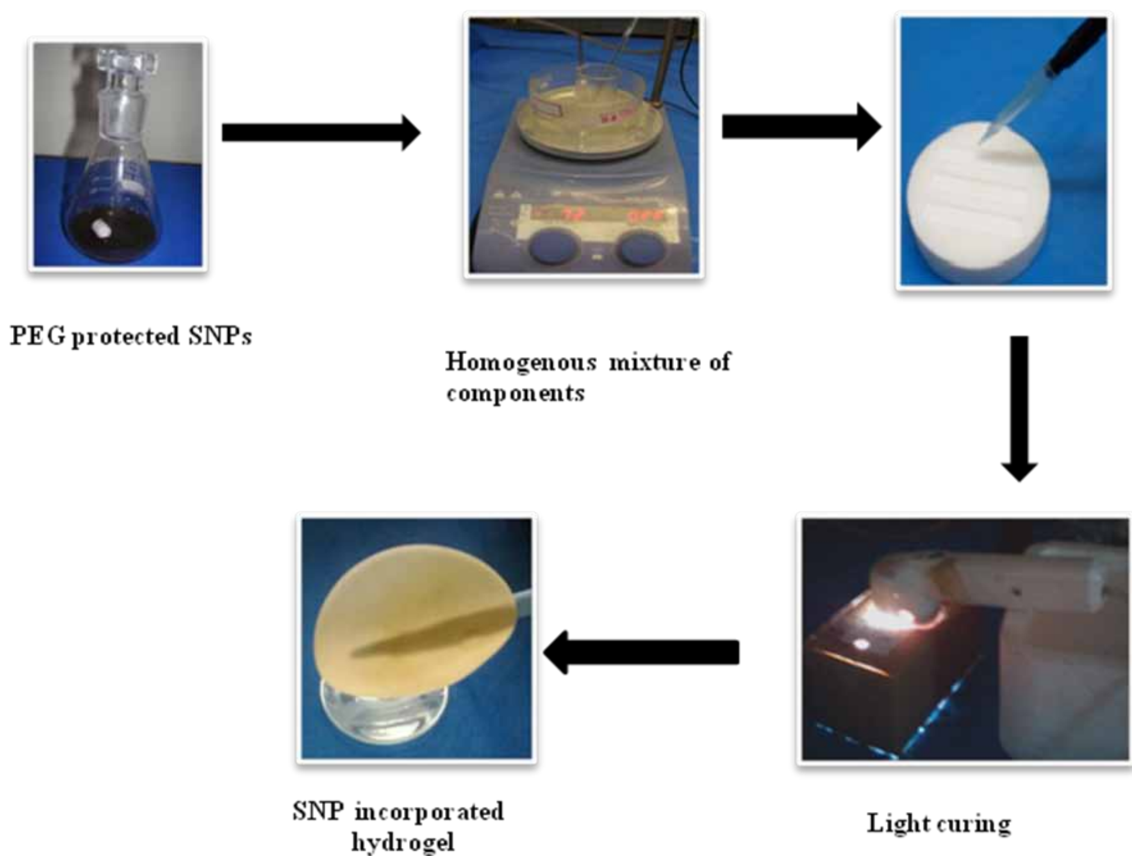

Figure 17. Fabrication of the SNP-incorporated hydrogel system

3.1.5. Fabrication of the Ofloxacin-incorporated hydrogel matrix

Fabrication of the ofloxacin-incorporated hydrogel matrix was carried out using the photo-polymerization method as explained in section 3.1.2. PCL and PEG were added to the HEMA monomer and heated to 70-75 ^{o}C until a homogenous mix was obtained. Antibiotic drug Ofloxacin was added to the mix followed by the addition of 1wt% photo-initiator (2, 4, 6 Trimethyl Benzoyl Biphenyl) Phosphine oxide (TPO) under

constant stirring. The solution was then poured into a Teflon mold (60 mm x 10 mm x 1 mm) and exposed to the dental light source to initiate the polymerization. Light exposure was provided for a maximum of 3-5 minutes. After completing the polymerization, the mold containing the cured polymer was kept in distilled water for up to 2 h to allow the polymer to peel away. Different formulations of drug-incorporated hydrogel systems were prepared and the details are given in Table 6.

Sample Code	**Wt %**			
	HEMA	**PCL**	**PEG**	**Ofloxacin**
Hdrg-0.25	85	10	5	0.25
Hdrg-0.5	85	10	5	0.5
Hdrg-0.75	85	10	5	0.75
Hdrg-1.0	85	10	5	1.0

Table 6. Formulations of drug-incorporated hydrogel systems

3.1.6. Preparation EGF-loaded hydrogel matrix –Solvent Sorption Method

pHEMA- PCL -PEG hydrogel was prepared as per the procedure discussed in section 3.1.2. Human recombinant EGF stock solution (100 µg) was dissolved in 1 ml sterilized PBS and loading was performed using the solvent sorption method. EGF stock solution in a concentration of 0.1 µg/µl was added drop by drop with a micropipette into the

hydrogels (~ 0.4 μg), 6 mm in diameter and 1mm in thickness. The samples were immediately transferred to a freeze-drier and lyophilized at -80 ^{o}C.

3.2. Characterization and evaluation of hydrogel systems

3.2.1. Structural characterization

Structural characterization of hydrogel was carried out using a Fourier Transform Infra Red spectrophotometer (Jasco FT-IR 6300, Japan) in the range of 4000-400 cm^{-1}. The lyophilized polymer samples were ground with KBr powder and the spectra were recorded as pellets.

3.2.2. Analysis of crystalline structure

The crystallinity of the hydrogel matrices was analyzed using an X-ray diffractometer. The prepared hydrogel samples were freeze-dried using a lyophiliser (Alpha 1-4 LD, Christ, Germany) and the XRD diffractogram was recorded using an X-ray diffractometer (Siemens D 5005, Germany) at 40 kV and 30 mA over a diffraction angle (2•) in the range of 10 to 80 ^{o}.

3.2.3. Swelling studies

With its capacity for fluid absorption, hydrogel provides a moist wound healing environment. To analyze the swelling properties of the polymer hydrogel samples, pre-weighed dried samples of dimension 8mm x 1mm were immersed in de-ionized water at 37 ^{o}C for 24 h. After removing the adherent water, the swollen samples were weighed at specific intervals of time using an analytical balance (Model CP224S, Sartorius,

Germany) with a sensitivity of 0.1 mg. The swelling percentage of the samples was determined through the following equation:

Percentage swelling, S% = ([Ws-Wo]/Wo)*100…….(1)

Where Ws and Wo are the masses of the swollen and dry samples, respectively.

3.2.4. Mechanical Properties

Evaluation of tensile strength and strain of hydrogels was carried out using a Universal Testing Machine (INSTRON Model 3365, UK) at 22 ^{o}C. Swollen hydrogel samples of 30 mm length, 10 mm breadth, and 1mm thickness were pre-conditioned before testing. A crosshead speed of 50 mm/min was maintained during the testing using a 100 N load cell. Tensile strength was calculated using the equation:

Tensile strength, MPa = Load in Newtons / Cross-sectional area in mm^2 .....(2)

Sample size was six per group, and the mean and standard deviation of the samples were calculated. Statistical analysis (ANOVA single factor) was carried out to determine significant changes in stress.

Figure 18. Tensile property evaluation using UTM

3.2.5. Surface wettability studies

Surface wettability of the dried hydrogel systems was determined via the sessile drop method using a video-assisted contact angle measuring device (DataPhysics OCA15 plus, Germany) and imaging software (SCA20 software,Germany). Within 10 seconds of introduction of the DI/W droplets, the contact angle formed between the sessile droplets and the matrix was measured. The contact angle is expressed as an average value of four independent measurements taken at different sites on each matrix.

3.2.6. Thermal analysis

The thermal properties of the hydrogel samples were studied using differential thermal analysis (SDT-Q600,TA instruments, USA) and differential scanning calorimetry (DSC instrument DSC-Q100, USA) as per ASTM 1131-08 and ASTM 3418-08 respectively . The temperature range of DTA analysis was room temperature (around 22 ^{o}C) to 600 ^{o}C at a heating rate of 10 ^{o}C. Thermogram was recorded (-100 to 150 ^{o}C) at a heating rate of 10 ^{o}C for DSC experiment.

3.2.7. Surface analysis

The surface topography of the hydrogel matrix was analyzed using a scanning electron microscope (Hitachi S2400, Japan). The dried hydrogel samples were coated with gold, placed on the aluminum stubs and observed under vacuum using a SEM. Environmental

scanning microscopy (ESEM, Quanta 200, FEI, Netherlands) was used to analyze the presence of EGF on the hydrogel matrix.

3.2.8. Water vapor transmission rate

The water permeability of the hydrogel matrix was determined by the procedure stipulated by the ASTM standard E96-00. The hydrogel matrix with a diameter of 30 mm was mounted on the mouth of glass vials (diameter of 28 mm), containing 10 ml of water with negligible water vapor transmittance. The material was fastened using Teflon tape in order to prevent water loss through the boundary and kept in an incubator at a temperature of 37 ^{o}C and relative humidity level of 35 %. The assembly was weighed at regular intervals of time and a weight loss versus time plot was constructed. WVTR was calculated using the formula:

$$WVTR = \frac{\text{Weight loss/time}}{\text{Area of specimen}} \text{ X } 24 \text{ g/m}^2\text{/day} \quad (3)$$

3.2.9. The release profile of SNP using ICP OES

Hydrogel systems (length-6 cm, breadth -1 cm and thickness -1 cm) , kept in 5 mLof de-ionized water for different time periods(24 h,48 h,72 h,96 h and 120 h) and deionized water were used as sample and blank respectively .Both the sample and blank were diluted to 10 mL with 2% HNO_3 and then analyzed using inductively coupled plasma optical emission spectroscopy (ICP/OES, Perkin Elmer, 5300DV,USA).The concentration of analyte element was determined from the calibration plot obtained by

analyzing standard solutions. The results were recorded and processed using Win Lab 32 software.

3.2.10. Antimicrobial activity testing

In vitro antimicrobial property evaluation of hydrogel systems incorporated with silver nanoparticle (HSN-3) and drug ofloxacin (Hdrg) was conducted using agar diffusion method. Susceptibility of bacterial strains to hydrogel discs was evaluated using the Zone of Inhibition Assay (ZIA). Bacterial strains used for the study were gram-positive (*S.aureus* ATCC 25923) and gram-negative (*E.Coli* ATCC 25922) respectively. Antimicrobial activity of hydrogel and SNP-incorporated hydrogel disc (6mm diameter and 1mm thickness) against Gram-positive and Gram-negative bacteria was investigated using gentamicin disc as positive control. Hydrogel system without SNP (HSN-3) of same dimension was used as negative control. The *in vitro* antibacterial test for drug incorporated hydrogel system was performed using gentamicin disc and ofloxacin disc as positive controls. Sample dimension of the test samples and controls were 6 mm diameter and 1mm thickness. The samples were placed on agar plates cultured with microbes at 37 ^{o}C for 24 h. The test was done in triplicate.

3.2.11. Encapsulation Efficiency of Ofloxacin

About 4 mg of powdered Hdrg was incubated with 100 ml of PBS for complete swelling. The mixture was transferred to a 50 ml centrifuging tube and agitated using a sonicator (Bandelin Sonorex Digitec ,Germany) for l0 minutes. After centrifugation (2000 rpm, 10 minutes), the supernatant solution was analyzed using UV-Visible spectroscopy

(Schimadzu, Japan) at a wave length of 290 nm. Drug encapsulation efficiency was calculated using the equation (4):

$$\text{Drug encapsulation efficiency} = \frac{\text{amount of drug present in the matrix}}{\text{theoretical amount of drug loaded}} * 100 \quad \ldots (4)$$

3.2.12. *In vitro* release profile of OFX drug

The total immersion method was used to study the *in vitro* release of the drug ofloxacin from the hydrogel systems. Hydrogel discs of 6mm diameter and 1mm thickness were punched out and incubated with 2 ml PBS buffer (pH 7.4) in a shaking incubator (IKA KS 4000 i, Germany) at 37 ^{o}C and 50 rpm. At defined time points, an equal volume of buffer was replaced with fresh buffer. The amount of ofloxacin in the sample solution was determined at the wavelength of 290 nm using a UV-Visible spectrophotometer. A calibration curve of ofloxacin was plotted in the range of quantity, within which a linear relationship between the concentration and absorbance was realized. The experiments were carried out in triplicates and reported as average values.

In vitro drug release from the hydrogel matrix can be explained using the expression derived from Fick's law (Equation 5).

$$F = Mt/M\infty = Kt^{n} \ldots\ldots\ldots (5)$$

Where F is the fraction drug release at time t, M_t and M_∞ are the amount of the drug released at time t and the maximum amount of drug released respectively. K is a kinetic constant characterizing the drug polymer interaction and the term n is the diffusional constant that characterizes the drug release transport mechanism.

3.2.13. *In vitro* release profile of EGF

EGF release from the loaded hydrogel matrices (H-EGF) was analyzed using a fluorescence-based assay. H-EGF discs of dimensions 6 mm x 1 mm were placed in Eppendorf tubes containing 2 mL PBS (pH=7.4). Release studies were carried out using a shaking incubator (50 rpm at 37 ^{o}C). At certain intervals, the released medium was replaced with fresh buffer. The release profile was analyzed using a fluorescence spectrophotometer (EL0507, Varian Cary Eclipse, Australia). EGF contains tryptophan residue, which exhibits intrinsic fluorescence at 280 nm excitation and 325 nm emission. A calibration curve of EGF was carried out in the range of 0.05 to 0.25 μgmL^{-1}. The cumulative release of EGF was calculated using the standard plot. The experiments were carried out in triplicates and reported as average values.

3.3. Biological Evaluation

3.3.1. Materials employed

Cell culture medium Dulbecco's Modified Eagle's Medium/Nutrient F12 Ham. (DMEM:HAM F12) and Foetal bovine serum were procured from Gibco BRL,USA . Antibiotic antimycotic solution (ABAM) was obtained from Invitrogen USA. Triton X-100, acridine orange and ethidium bromide were purchased from Sigma Aldrich, USA.

Phalloidin conjugated with Texas red was obtained from Molecular Probes, USA. ^{3}H-thymidine was procured from American Radiochemicals, USA.

3.3.2. Percentage Hemolysis Test

Hemocompatibility testing of hydrogel systems was carried out broadly on the basis of ISO 10993-4:2002 (E) selection of tests for interaction of materials with blood. The test is mainly aimed at finding the extent of hemolysis caused by the sample. Blood from a healthy human volunteer was collected and the anticoagulant CPD-A was added as required. The samples kept in PBS were taken out and placed in polystyrene plates. Two ml of blood was added to each sample, one ml blood was taken immediately for initial analysis and 1ml blood was incubated with the samples for 30 min under agitation at 70 ± 5 rpm using an Environ shaker thermostated at 35 ± 2 ^{o}C. Four empty polystyrene culture dishes were exposed with blood as reference. The total hemoglobin in the whole blood samples was measured using an automatic hematology analyzer (Sysmex-K 4500). The free hemoglobin liberated into the plasma after exposure to materials was measured using a diode array spectrophotometer (HP 8453 Hewlett-Packard GmbH, Germany) and the hemolysis percentage was calculated using the following formula:

% Hemolysis= (Free Hb/Total Hb) X 100………..(6)

3.3.3. Cell culture of human skin fibroblasts in presence of hydrogel matrices

Human skin fibroblasts isolated from foreskin was used for the study. The cells were supplied by Thrombosis Research Unit for this study. Frozen cells (passage 3 to 6) were

thawed and known density of cells was seeded on the polystyrene culture dishes. Media used was DMEM:HAM F12 containing 10% fetal bovine serum (FBS) and 1X antibiotic antimicotic (ABAM) solution. Culture was maintained and the media was changed on alternate days, and expanded when it reached 80-90 % confluence. When cells attained 90% confluence, cells were exposed to the material (HI-3, HSN-3, Hdrg, and H-EGF) by direct method, exposure time varying from 24 h to 72 h. *In vitro* cytotoxicity, cell viability, cell adhesion and proliferation assay were then carried out to understand the effect of materials on the cell growth and proliferation as detailed below.

3.3.3.1. In vitro Cytotoxicity by direct contact assay

An *in vitro* cytotoxicity test using the direct contact method was performed using fibroblast. The test samples were immersed in culture medium containing serum for 30 min prior to testing. Samples were placed on the cells (40000 cells/cm^2) and medium was replaced with fresh medium. After incubation at 37± 1 ^{o}C for 48 h, the cell monolayer was imaged using optical microscope and Leica Application Suit software (Leica, DMIRB,Germany) at a magnification of 10X.

3.3.3.2. Analysis of cell viability

Cell viability was analyzed by live-dead assay using acridine orange and ethidium bromide. Briefly, the fibroblasts were cultured for 48h in direct contact with the hydrogel matrix at 37 ^{o}C in a humidified CO_2 incubator in DMEM: HAM F12 medium containing 10% FBS. After 48 h, the hydrogel was removed and the cells were washed with sterile PBS (thrice). Cells were incubated with acridine orange (1:100 dilution or 0.1 µg / ml)

and ethidium bromide (1:100 dilution 0.1 μg / ml) in PBS at room temperature for 20 min. Cells were washed with PBS thrice at 10-min intervals and images were captured using a fluorescent microscope and Leica Application Suit software (Leica, DMIRB,Germany) at a magnification of 10X .

3.3.3.3. Analysis of cell proliferation

Fibroblast proliferation in the presence of hydrogel systems was carried out using the ^{3}H-thymidine uptake assay. Fibroblasts in log phase were labeled with ^{3}H-thymidine by adding the nucleotide at a concentration of 10 μCi ml^{-1} in the culture media which was then cultured for one week at 37°C and 5% CO_2 to achieve more than two population doublings. Trypsinized ^{3}H-thymidine-loaded cells (5.4 x 10^4 cells) were cultured in contact with hydrogel samples (HI-3) in DMEM: HAM F12 medium containing 10 μCi ml^{-1} ^{3}H- thymidine for 48 h at 37 ^{o}C and 5% CO_2 . The radioactivity of an aliquot containing 5.4 x 10^4 of cells was counted using Triathler Multilabel Tester (Hidex, Finland). The cells were harvested after 48 h and radioactivity of ^{3}H- thymidine was counted. The number of cells after 48h from each well was calculated from the radioactivity of the known number of cells. Doubling time was calculated using an online programme (http://www.doubling-time.com/compute.php).

3.3.3.4. Cell Adhesion study

Cells were seeded at a density of 70000 cells /cm^2 in culture wells and HI-3 samples were kept on the cells. Cells in polystyrene culture plates served as control. Cells were allowed to grow for 72 h. After 72 h, hydogel was taken out and the cells in control dish, the material taken out as well as cells in dishes which contained the material were fixed with

3.7% formaldehyde for 20 min and then permeabilised with 0.2% triton X 100. Texas Red-conjugated antibodies (1:500) against actin cytoskeleton were added and kept at 37 ^{o}C for 30 minutes, washed thoroughly using PBS and observed through fluorescent microscope (Leica DMIRB, Germany) at a magnification of 20X.

3.5. Statistical Analysis

Three to six measurements were done for each analysis and the quantitative data are presented as average ± standard deviation. ANOVA single factor was taken for comparison purposes in which $p < 0.05$ was used as the criterion for significant difference. The number of tests for each parameter is indicated in the respective figure legends.

CHAPTER 4

RESULTS

Chapter 4 contains the results of the current study. It is divided into four subsections. The first section details the fabrication of the pHEMA-PCL-PEG hydrogel matrix and optimization of its properties. The second section discusses the fabrication and characterization of the antimicrobial hydrogel matrices using silver nanoparticles and section three using antibiotic ofloxacin. The fourth section describes the incorporation of the epidermal growth factor into the hydrogel matrix and its potential as a wound dressing material.

4.1. Fabrication and characterization of hydrogel matrices

The main objective of this section is to discuss the development of the hydrogel systems using different compositions of HEMA, PCL and PEG and the optimization of the compositions based on the hydrogel properties.

4.1.1. Fabrication of hydrogel matrix

Figure 19. PHEMA-PCL-PEG hydrogel sheet

PHEMA hydrogel sheets containing PCL and PEG were successfully fabricated via the photo-polymerization technique. Figure 19 shows the hydrogel in lyophilized form. Schematic representation of the hydrogel formation is shown in Figure 20.

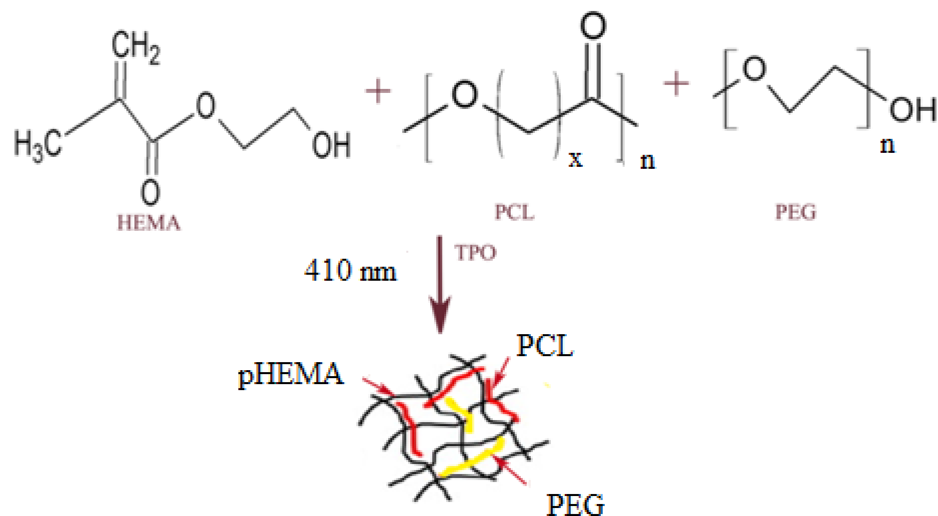

Figure 20.Schematic representation of formation of hydrogel

4.1.2. Characterization of the hydrogel systems

4.1.2. 1. Structural characterization using FT-IR spectroscopy

Structural characterization of the hydrogel systems was done using FT-IR spectroscopy and the spectra were compared with that of the monomer HEMA (Figure 21A). The HEMA monomer showed strong absorption 1636 cm^{-1}, 1726 cm^{-1} and 3530 cm^{-1} while the hydrogel systems showed absorption at 1163 cm^{-1} ,1727 cm^{-1} and 3533 cm^{-1} . Absorption at 1636 cm^{-1} was found to be absent in the spectrum all hydrogel systems (Figure 21B).

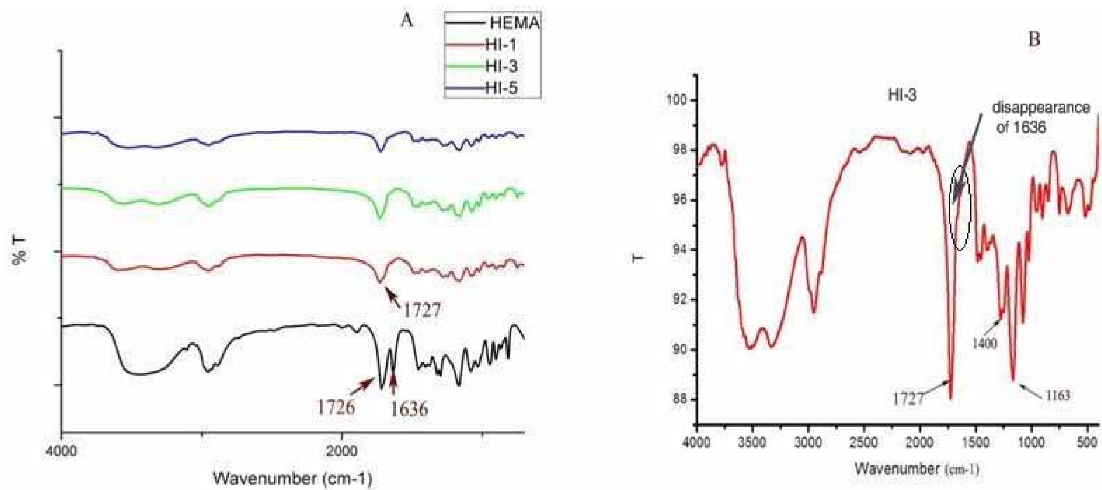

Figure 21. FT–IR spectra of (A) HEMA monomer, Hydrogel systems HI-1, HI-3 and HI-5 (B) HI-3 alone

4.1.2. 2. Analysis of crystalline structure

The crystalline/amorphous nature of the hydrogel systems was evaluated using XRD. Figure 22 shows the XRD patterns of the pHEMA hydrogel and pHEMA/PEG/PCL hydrogel systems. The pHEMA hydrogel did not exhibit any diffraction peak while HI-1 to HI-6 systems showed strong reflection peaks at 21.40 ^{o} and 23.71 ^{o}.

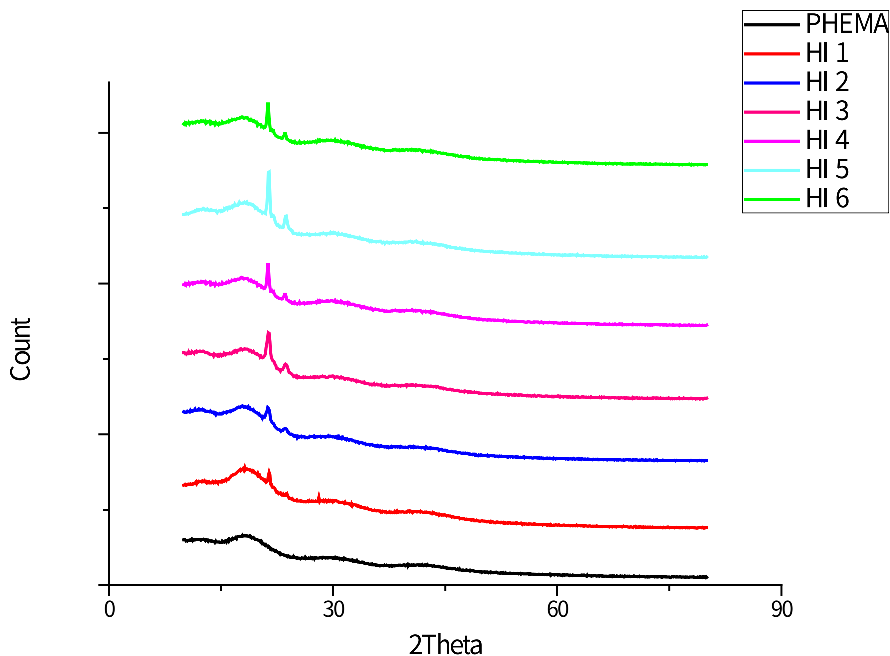

Figure 22. XRD patterns of pHEMA and combination systems

4.1.2.3. Swelling studies

The swelling capacity of the hydrogel systems in distilled water is shown in Figure 23. All hydrogel systems were found to possess swelling capacity of more than 50%. The highest value of swelling was exhibited by Hydrogel HI-3 (64 ± 2.8 %), while HI-5 showed lowest value (52 ± 1%).

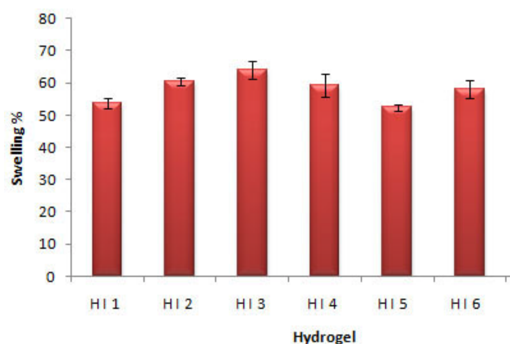

Figure 23. Swelling capacity of hydrogels

4.1.2.4. Mechanical properties

The tensile strength and elongation of the synthesized hydrogel systems are shown in Figures 24 and 25. HI-3 showed the highest tensile strength (0.23± 0.03 MPa) and good elongation (111± 17 %).

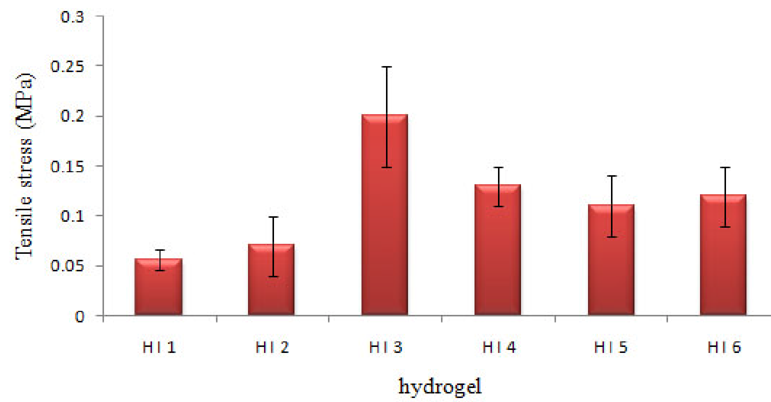

Figure 24. Tensile strength of hydrogel systems

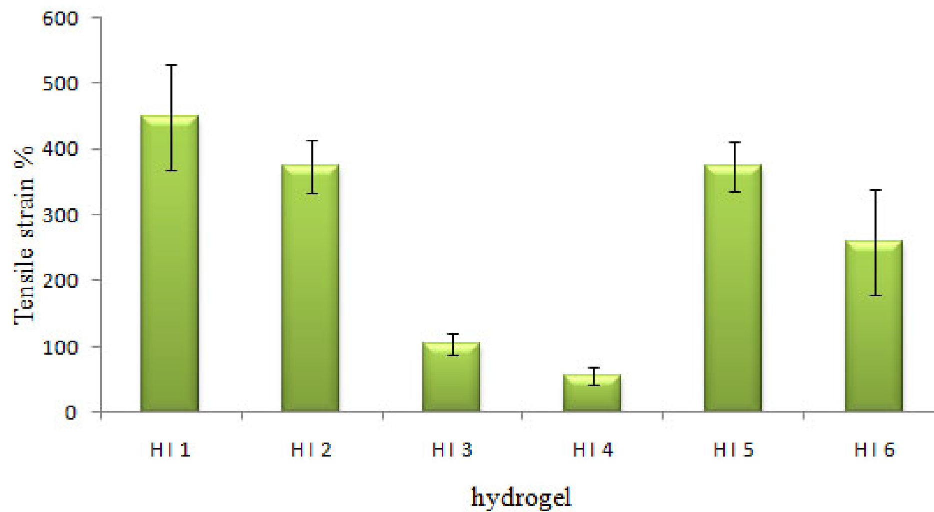

Figure 25. Tensile strain of hydrogel systems (n• 3)

4.1.2.5. Optimization of hydrogel composition

For an ideal wound dressing material, good handling characteristics as well as a moist surface with excellent water vapor transmission rate is a prerequisite. Therefore based on the swelling and mechanical property evaluation, the hydrogel system HI-3 with 64% swelling capacity and possessing better mechanical properties out of the 6 systems studied was selected for further studies.

4.1.2.6. Surface Wettability Studies

Surface hydrophobic/hydrophilic character of the hydrogel system was analyzed by measuring the contact angle using sessile drop method (Figure 26). A mean contact angle value of 54 ± 1° indicated high hydrophilic nature of the system. Table 7 shows the values of contact angles (n=6) obtained for systems studied.

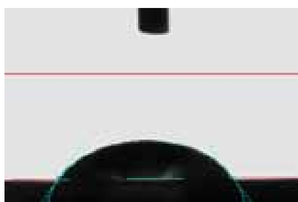

Figure 26. Representative image showing surface wettability of hydrogel matrix HI 3

Sample	Contact angle (θ)
HI 3	54 ± 1°
PHEMA	$< 20^{o}$
PCL	73°

Table 7. Surface wettability characteristics

4.1.2.7. Thermal Analysis

Thermal stability of HI-3 using TGA analysis (Figure 27) exhibited initial thermal decomposition at 196.82 ^{o}C and 50% mass loss at 367.7 ^{o}C. Final decomposition of the polymer was achieved at 573.89 ^{o}C. The DSC thermogram of HI-3 showed only a single glass transition temperature (Tg) at – 27.8 ^{o}C. Melting of the hydrogel is observed to occur at 51.85 ^{o}C (Figure 28).

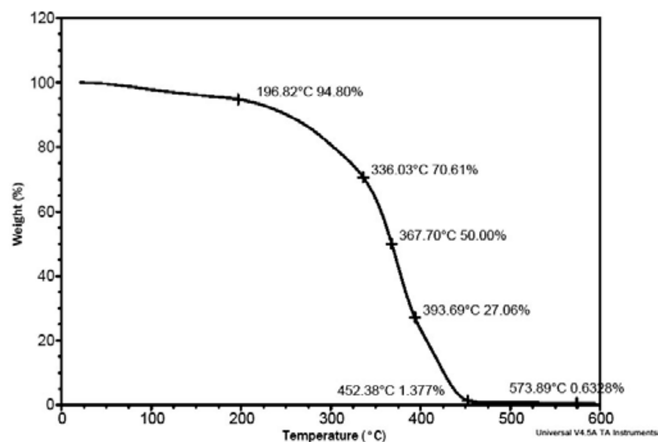

Figure 27. TGA thermogram of HI-3

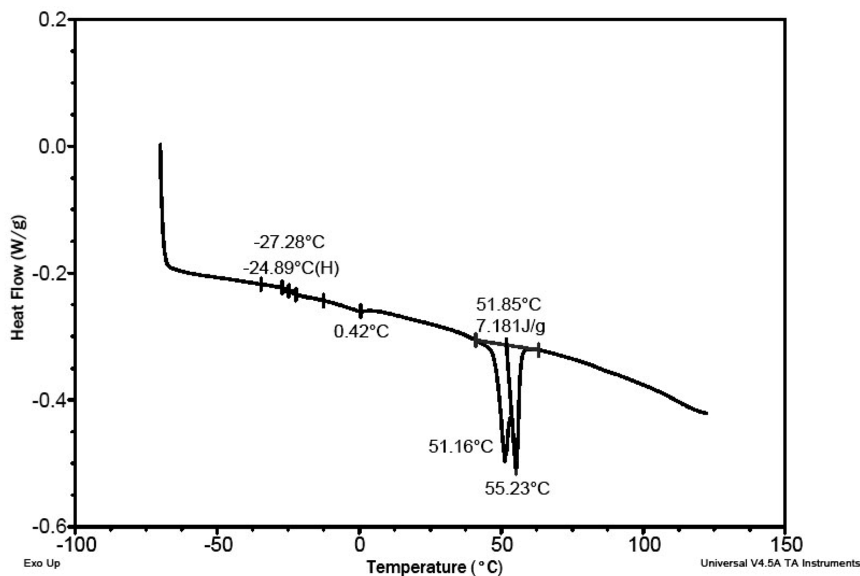

Figure 28.DSC thermogram of HI-3

4.1.2.8. Surface Analysis

Surface morphology analysis of the HI-3 hydrogel surface in the lyophilized state using scanning electron microscopy revealed a porous cross-sectional layer (Figure 29 a) and a dense outer layer and (Figure 29b)

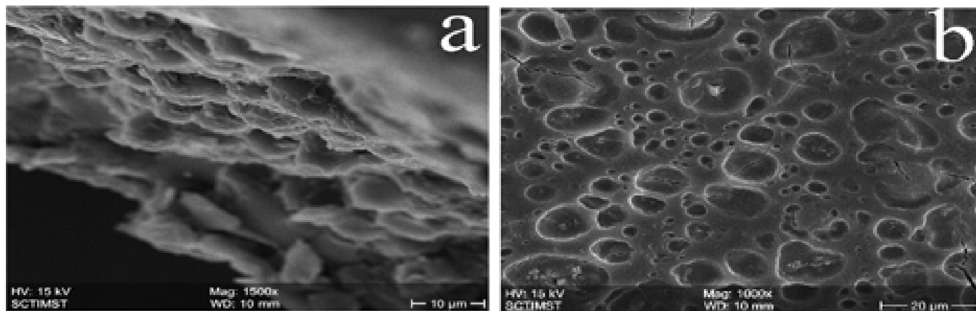

Figure 29. SEM micrographs of HI-3 (a) cross section (b) surface

4.1.2.9. Water vapor transmission rate (WVTR)

Water vapor transmission is one of the most important properties of wound dressing material, as it controls the wound dehydration to a great extent. In the present study, WVTR was calculated as the gradient of weight loss with time. Table 8 shows the loss of water vapor through the hydrogel matrix HI-3 when placed in a moisture environment. WVTR of HI-3 was compared with that of normal skin, first degree burn wounds, granulating wounds and commercially available dressings.

Sample	WVTR ($g/m^2/day$)
Normal skin	204 ± 12
First degree burn	279 ± 29
Granulating wound	5138 ± 202
Wound dressing	1900-2500
HI-3	2010 ± 100

Table 8. Comparative data of WVTR

4.1.3. Biological Evaluation of Hydrogel Matrix

4.1.3.1. Hemolysis study

The effect of HI-3 hydrogel upon % hemolysis was studied in order to make sure that no adverse effects are observed. An ideal wound dressing material is expected to produce hemolytic effect only within permissible limits (< 5%) at the wound site. Procedure stipulated as per ISO standard 10993-4:2002 (E) was followed in order to determine the hemolytic property of the hydrogel (Table 9).

Sample	% Hemolysis
Polystyrene culture dish (control)	0. 04±0.01
HI-3	0. 08±0.01

Table 9. Analysis of percentage hemolysis (n• 3)

4.1.3.2. *In vitro* cytotoxic evaluation

4.1.3.2.1 Cytotoxicity by direct contact assay

Results of the direct contact test between the fibroblasts and HI-3 are shown in Figures 30A and B. Fibroblasts in contact with HI-3 showed non-cytotoxic behavior and found to

retain their spindle-shaped morphology (Figure 30 B) in comparison with the control (cells grown on tissue culture plates) (Figure 30 A).

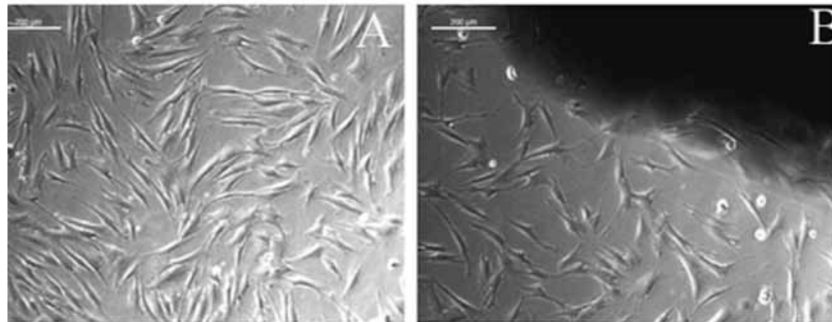

Figure 30. Microscopic images of fibroblast in A) culture plate B) contact with HI-3 (n• 3)

4.1.3.2.2. Cell Viability Assay

Figures 31 A and B depict the microscopic images of control fibroblasts and fibroblasts incubated with HI-3. Cell viability of the fibroblast in presence of HI-3 was analyzed using acridine orange and ethidium bromide staining. Green color indicates live cells. Cell count was done using Image A software and cell viability was found to be nearly 100%.

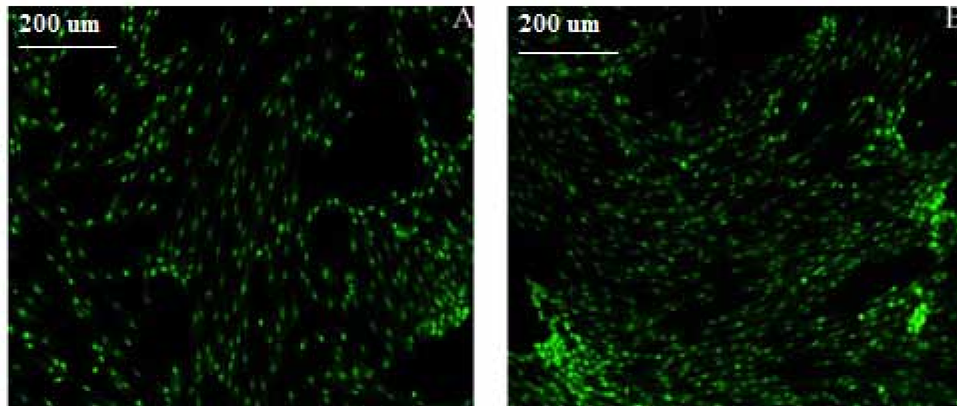

Figure 31. Live-dead assay using acridine orange and ethidium bromide on fibroblast, incubation at 37^{0}C for 48h (A) control (B) HI-3 (n• 3)

4.1.3.2. Cell Adhesion Study

Texas Red staining of the actin filaments was carried out to evaluate the cell adhesion property of the hydrogel matrix. Microscopic observation of the hydrogel surface in contact with the fibroblast indicated non-adherence of the cells to the material (Figure 32 B). Cell morphology as well as spreading underneath the hydrogel (Figure 32 C) was found to be comparable with the medium (Figure 32 A).

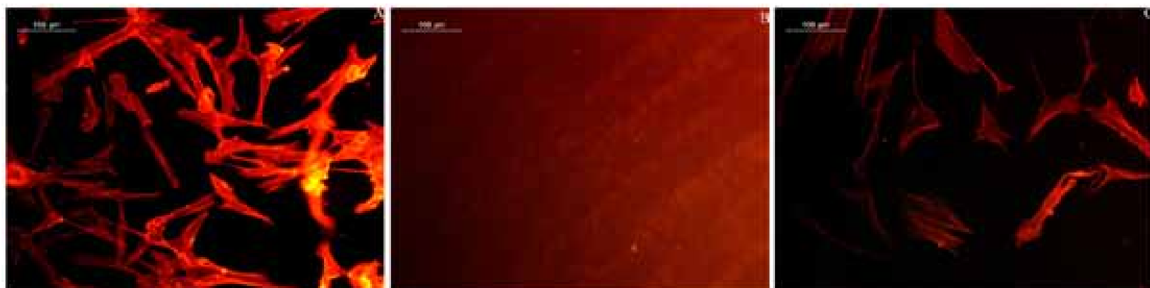

Figure 32.Microscopic images of actin staining on A) control cells B) HI-3 C) fibroblast in contact with HI-3 (n• 3)

4.2. Fabrication and characterization of silver nanoparticle incorporated antimicrobial hydrogel systems

4.2.1. Fabrication of SNP-incorporated hydrogel matrix

Incorporation of silver nanoparticle to the HI-3 hydrogel system was carried out using photo-polymerization technique .Successful incorporation of SNPs into the matrix was evidenced by the color change of the matrix (Figure 33).

Figure 33. SNP-incorporated hydrogel matrix

4.2.2. Characterization of SNP

4.2.2.1. Transmission electron microscopy and UV spectroscopic Analysis

Silver nanoparticles were synthesized and protected from aggregation using polyethylene glycol (*Madhavan et al*., 2011).Transmission electron micrographs showed spherical particles of < 20 nm size (Figure 34A) and UV-Visible spectra analysis revealed a characteristic peak at 410 nm (Figure 34B).

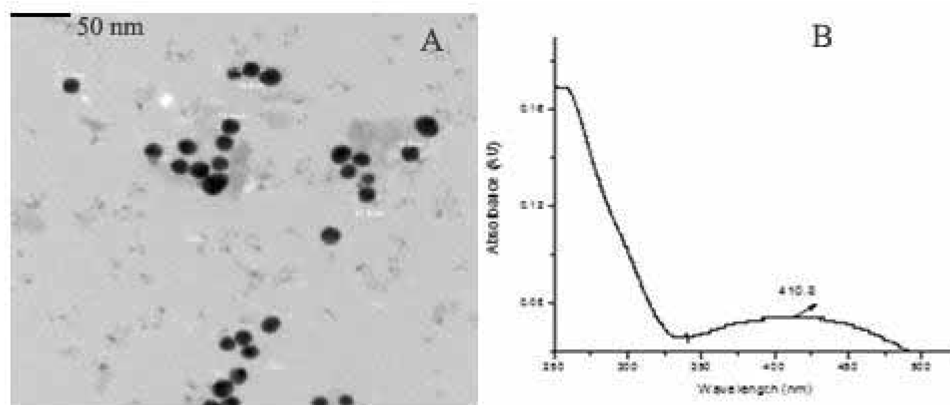

Figure 34. (A) TEM image (B) UV-Vis spectrum of SNP

4.2.2.2.Release profile of SNP from the matrix

In vitro release of SNPs from the matrix was analyzed using inductively coupled plasma optical emission spectroscopy (ICP- OES) technique. The release profile of SNP from the ICP-OES analysis of HSN-3 in 4 days is depicted in Figure 35. Nearly 75% of the silver particles were found to be eluted from the hydrogel matrix within 4 days of immersion.

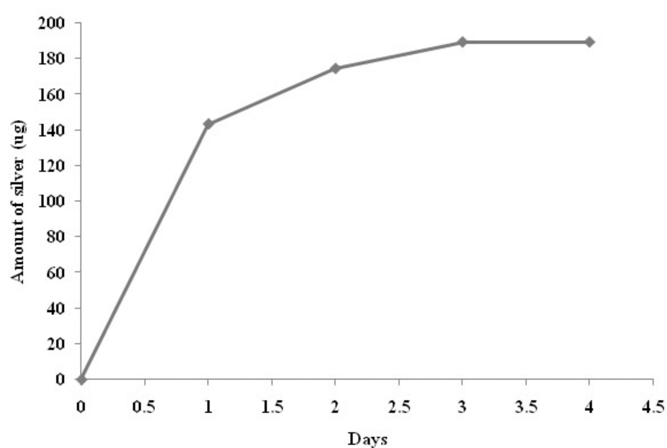

Figure 35. Release profile of SNPs using ICP-OES (n=3)

4.2.2.3. Swelling analysis of HSN-3

In order to evaluate the effect of SNPs in the water uptake ability of hydrogel systems, swelling analysis was carried out in distilled water and was compared. The swelling ability of HSN-3 is depicted in Figure 36. HSN-3 showed less water intake (35 %) when compared to HI-3 (65%).

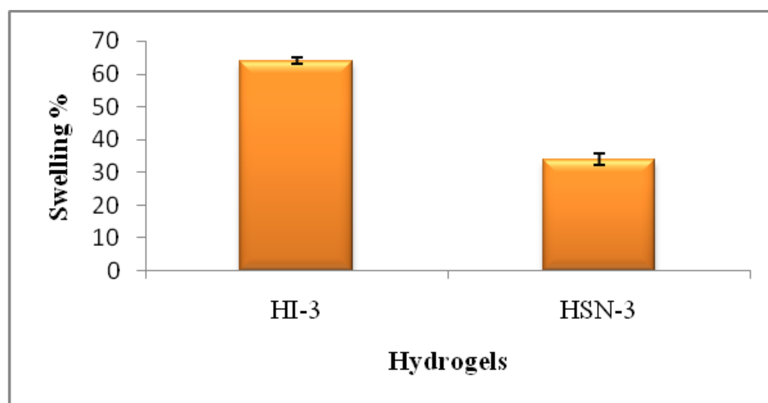

Figure 36. Swelling capacity of the hydrogel systems (n• 3)

4.2.2.4. Surface Wettability Studies

Effect of silver nanoparticle on the surface wettability of hydrogel system was evaluated using contact angle study (Figure 37) .Using the sessile drop method, surface hydrophilicity of HSN-3 was compared with that of hydrogel system without SNPs. The contact angle of HSN-3 was found to be 56 ± 0.15^{0}.

Figure 37. Surface wettability of HSN-3

4.2.3. Biological Evaluation of HSN-3

4.2.3.1. Hemolysis Study

Hemolysis analysis was conducted to find out the effect of SNPs on hemolysis at the wound site. % hemolysis analysis was conducted as per the ISO standard 10993-4:2002 (E). It was observed that HSN-3 produces a percentage hemolysis of about 0.16 ±0.03%.

4.2.3. 2. *In vitro* cytotoxic evaluation

4.2.3.2.1. Cytotoxicity by direct contact assay

Direct contact test was conducted to study the cytotoxic effect of hydrogel matrix on fibroblast. Figures 38 A, B and C depict the microscopic images of fibroblasts grown in culture plate (cell control), fibroblasts in presence of HI-3 and fibroblasts incubated with HSN-3 respectively. *In vitro* cytotoxic evaluation indicated that the fibroblasts in contact with HSN-3 retained their spindle-shaped morphology (Figure 38 C) which is comparable with that of the control cells (Figure 38 A).

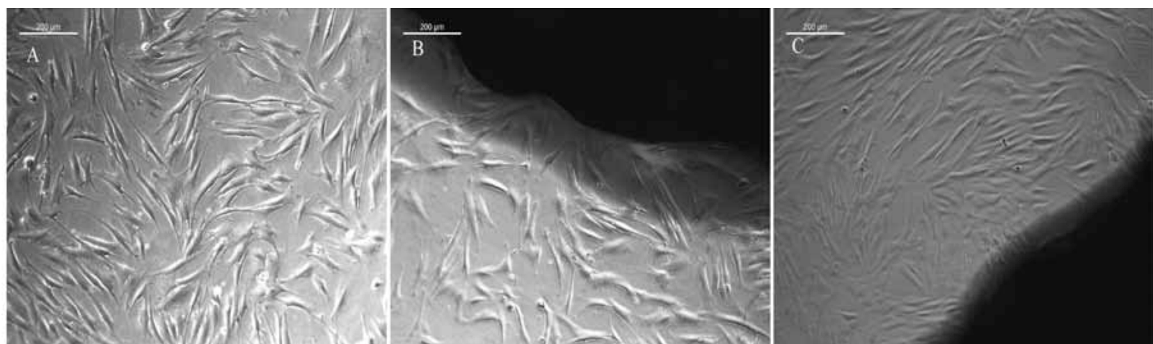

Figure 38. Fibroblasts in direct contact with (A) cell control (B) HI-3 (C) HSN-3 for 48h (n• 3)

4.2.3.2.2. Cell viability

Live-dead assay using acridine orange and ethidium bromide staining was conducted to analyze the cell viability in presence of HSN-3. Fibroblasts grown in culture plate was used as the control in the study .Figures 39 A and B shows the fluorescent microscopic image of the control cells and fibroblasts incubated with HSN-3. Green color indicates live cells.

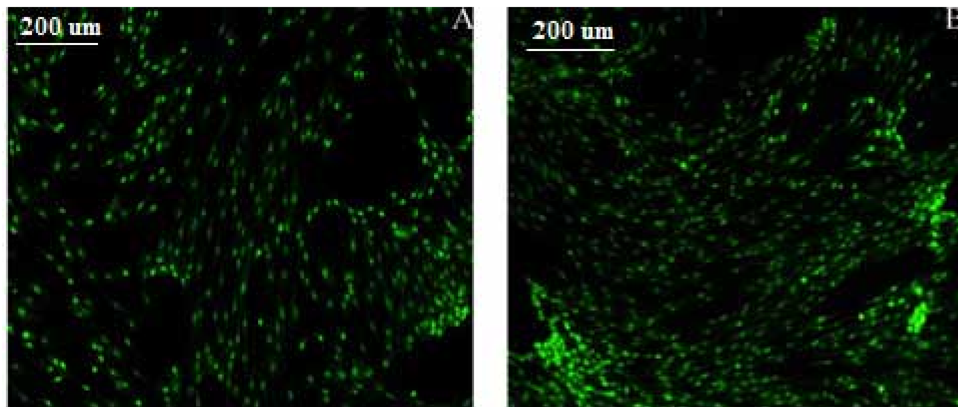

Figure 39. Live-dead assay using acridine orange and ethidium bromide on fibroblasts, incubation at 37^{0}C for 48h (A) control (B) HSN-3(n• 3)

4.2.3. 3. Cell Adhesion Study

Figures 40 D ,E & F depict the fluorescent microscopic images of actin staining on control cells, hydrogel matrix in contact with fibroblasts and fibroblasts grown underneath the hydrogel respectively. Texas Red staining of the actin filaments showed the fibroblasts to be non-adherent to the material HSN-3 (Figure 40 E). Cell morphology as well as cell spreading underneath the hydrogel HSN-3 (Figure 40 F) was found to be comparable with that of control (Figure 40 D).

Figure 40. Microscopic images of actin staining on (D) Control fibroblasts (E) HSN-3 hydrogel (F) Fibroblasts in contact with HSN-3 (n• 3)

4.2.3.4. Evaluation of Antimicrobial Activity

Agar disc diffusion method was used to analyze the antimicrobial property of the SNP incorporated hydrogel matrix. The antimicrobial property of the HSN-3samples was evaluated against gram-positive (*S.aureus* ATCC 25923) and gram-negative (*E.Coli* ATCC 25922) bacteria (bacterial count 10^4/ml). HSN-3 was found to be protective against both gram-positive and gram-negative bacteria. Gentamicin disc (positive control) showed a large zone of inhibition against both strains while the bare disc and HI-3 (negative control) did not exhibit any zone of inhibition.

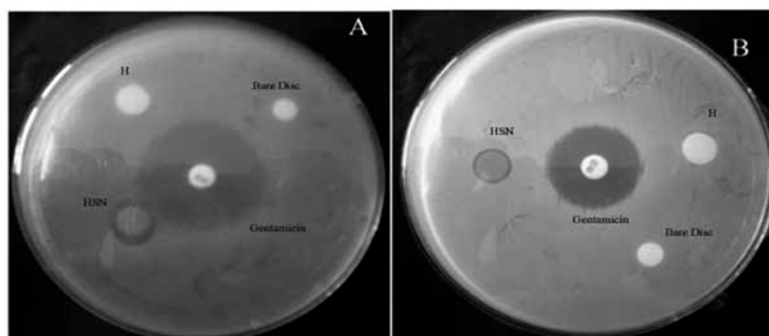

Figure 41. Representative patterns of zone of inhibition assay : Antimicrobial activity of HSN-3 against (A) *Escherichia Coli.* (B) *Staphylococcus aureus* (n• 3)

4.3. Fabrication and characterization of the Ofloxacin-incorporated hydrogel matrix (Hdrg)

4.3.1. Fabrication of the Ofloxacin-incorporated hydrogel matrix

In order to impart antimicrobial property to the HI-3 hydrogel system, an antibiotic drug ofloxacin was used in the study. The photo-polymerization technique was effectively used to prepare the ofloxacin-incorporated hydrogel matrix (Figure 42).

Figure 42. Ofloxacin-incorporated hydrogel matrix (Hdrg)

4.3. 2. Characterization of Ofloxacin-incorporated hydrogel matrix

4.3.2.1. Swelling analysis

To analyze the effect of incorporated drug on the fluid uptake ability of hydrogel system, swelling analysis was carried out in distilled water. The results of the swelling analysis of hydrogel systems are depicted in Figure 43. Comparative evaluation of swelling capacity indicated that fluid uptake ability of Hdrg1 (40 ± 2%) was found to be significantly lower than that of HI-3.

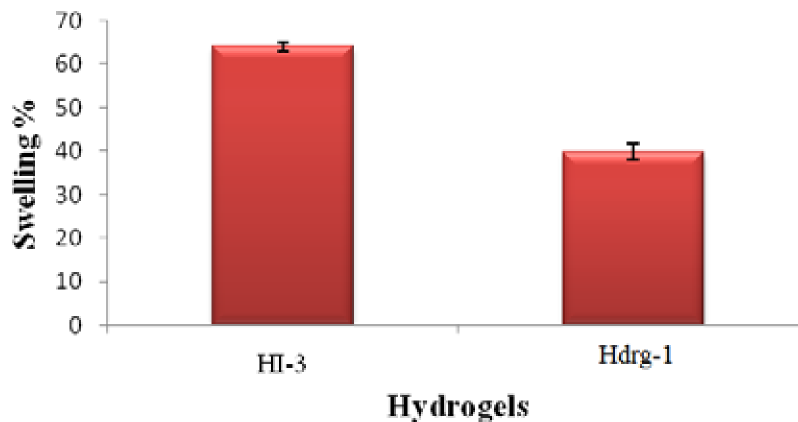

Figure 43. Swelling capacity of hydrogel systems (n• 3)

4.3.2.2. Surface Wettability Study

Effect of drug incorporation in the surface hydrophilic property of the hydrogel matrix was evaluated by measuring contact angle using sessile drop method. The observed value of the contact angle of Hdrg1 was found to be $61 \pm 2\ ^{o}$. It was observed that addition of drug increased the surface hydrophobicity of the matrix.

4.3.2.3. Drug Encapsulation Efficiency

Evaluation of drug encapsulation efficiency is an important aspect which provides a quantitative estimation of the actual drug within the hydrogel after formulation. UV spectroscopy technique was used for this study. Percentage encapsulation efficiency of ofloxacin in the hydrogel system was found to be nearly 60%.

4.3.2.4. *In vitro* release study of ofloxacin

4.3.2. 4.1. Effect of medium

A drug release study was performed in distilled water and PBS (pH=7.4). Faster drug release was observed in PBS compared to water. The complete release of the drug from

the matrix took nearly 144h in distilled water, whereas in PBS, the initial burst release was observed in the first 24 hrs followed by sustained release for 144 h (Figures 44 A and B).

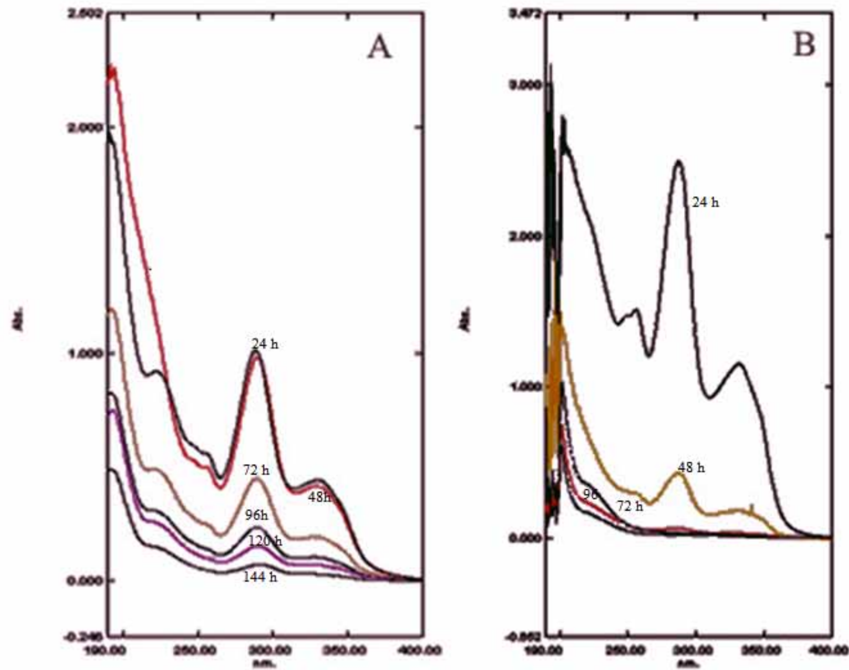

Figure 44. Release study of ofloxacin A) water B) PBS

4.3.2. 4.2. Effect of drug loading

Figure 45 displays the release profile of ofloxacin from hydrogels at different amounts of drug loading. The data show that the hydrogel systems containing the highest amount of the drug exhibited a faster release rate than that of the systems with less drug loading. Hdrg1 showed a faster rate of drug release while Hdrg 0.25 displayed a slower rate of drug release.

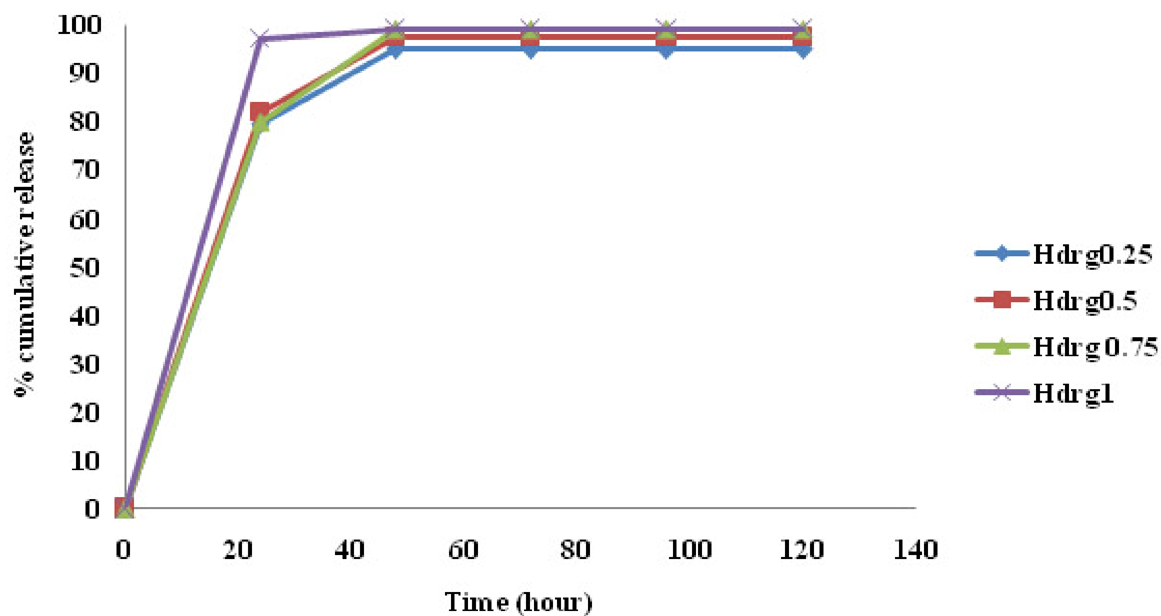

Figure 45. Cumulative release of drug from hydrogel systems with different drug loadings (n• 3)

4.3.2. 4.3. Drug release kinetics

Release kinetics was analyzed using the cumulative release data vs. time (Figure 45) by fitting to an exponential equation (Equation 5). Drug release kinetics parameters k and n of different formulations are shown in Table 10.

Sample code	k	n
Hdrg-1	0.601	0.823
Hdrg-0.75	0.639	0.738
Hdrg-0.50	0.647	0.7226
Hdrg-0.25	0.635	0.720

Table 10. Drug release kinetic parameters of different drug loading

4.3.3. Biological Evaluation of Hdrg

4.3.3.1. Hemolysis Study

Hydrogel system without drug was found to be non hemolytic. To study the effect of drug incorporation on hemolytic property of hydrogel system, % hemolysis assay was conducted as per the ISO standard 10993-4:2002 (E) and the % hemolysis was found to be 0.156± 0.03%.

4.3.3.2. *In vitro* cytotoxic evaluation

4.3.3.2.1. Cytotoxicity by direct contact assay

Cytotoxic evaluation of the hydrogel system with higher drug loading (Hdrg-1) was performed using direct contact assay. Figures 46 A, B and C show the microscopic images of control fibroblasts, fibroblasts incubated with HI-3 and Hdrg-1 respectively. Incubation of the fibroblasts with Hdrg1 for 48 h did not have any undesirable effect on the cells as evidenced by retention of spindle-shape morphology as well as good spreading (Figure 46 C).

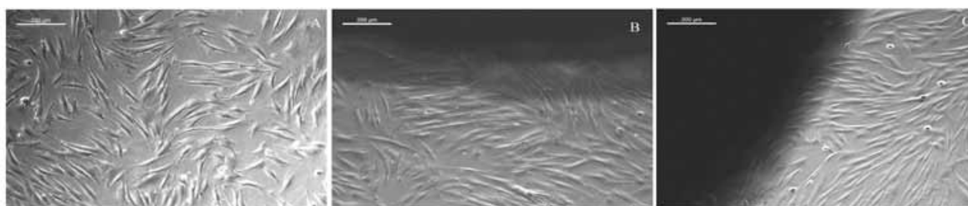

Figure 46. Microscopic image of fibroblasts (A) control cell (B) contact with HI-3 (C) With Hdrg1 (n• 3)

4.3.3.2.2.Cell viability

Acridine orange and ethidium bromide staining on fibroblast after incubation with Hdrg1 for 48 h was conducted to evaluate the cell viability. Figures 47 A and B displays the fluorescent microscopic images of control fibroblasts and fibroblasts incubated with

Hdrg-1. Cell counting was carried out using Image J software and nearly 100% of the cells were found to be viable after incubation with Hdrg-1 for 48h.

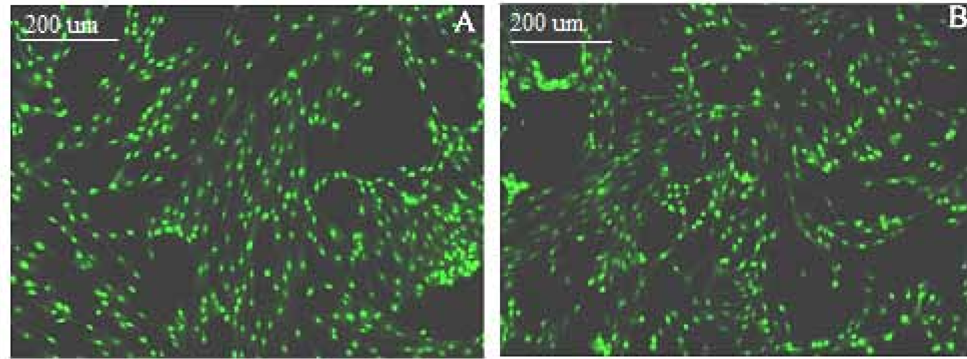

Figure 47. Live-dead assay using Acridine Orange and Ethidium Bromide on fibroblasts, incubation at 37 °C for 48 h (A) control (B) Hdrg-1 (n• 3)

4.3. 3. 3. Cell adhesion study

Non adherence to fibroblast cell is found to be an important quality of the wound dressing material. Figures 48 G, H and I depict the microscopic images of actin staining on control fibroblast, Hdrg1 hydrogel and fibroblast in contact with Hdrg1 respectively. Cell adhesion study was conducted using actin staining exhibited non - adhesion of the fibroblasts to Hdrg1 (Figure 48 H) while the fibroblast grown underneath the hydrogel Hdrg-1 exhibited good spreading and spindle shaped morphology (Figure 48 I).

Figure 48. Microscopic images of actin staining on (G) Control fibroblast (H) Hdrg hydrogel (I) Fibroblast in contact with Hdrg-1 (n• 3)

4.3.4. Antimicrobial property evaluation

The antimicrobial property of the hydrogel matrix with different drug loadings (Hdrg - 0.25, Hdrg-0.5, Hdrg-0.75 and Hdrg-1) was evaluated against gram-positive (*Staphylococcus aureus*) and gram- negative (*Escherichia coli)* bacteria using the agar disc diffusion method. Figure 49 displays a clear inhibitory zone around Hdrg against *Escherichia Coli* and *Staphylococcus aureus*. The zone of inhibition of Hdrg was found to be comparable with that of the gentamicin control (Table 11). Figure 50 represents the zone of inhibition of the Hdrg systems (Hdrg-0.25, Hdrg-0.5 and Hdrg-0.75) in comparison with ofloxacin and it was observed that Hdrg systems exhibited a clear zone of inhibition of against gram-positive and gram-negative bacteria and was found to be comparable with that of the ofloxacin disc (Tables 12 and 13).

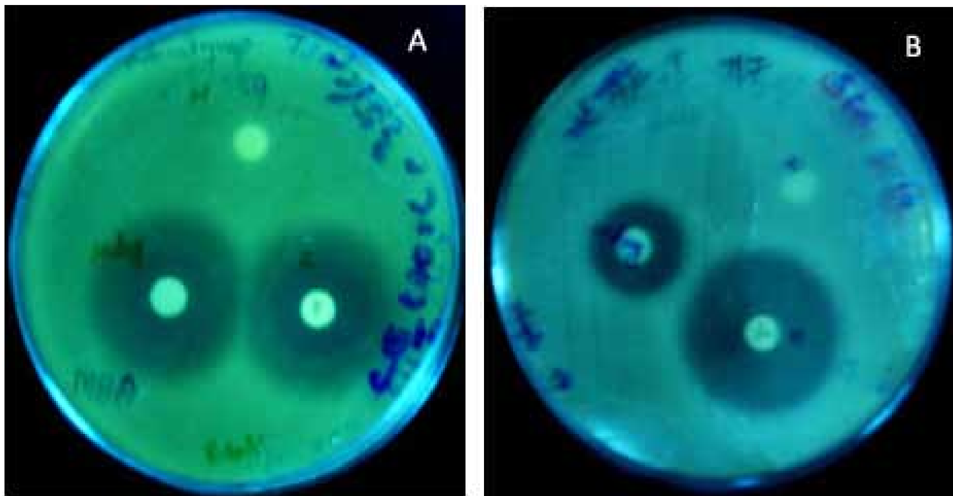

Figure 49. Representative patterns of zone of inhibition assay of Hdrg1 against (A) ***Escherichia Coli.*** **(B)** ***Staphylococcus aureus*** **(n• 3)**

Samples	Zone of inhibition	
	E.Coli ATCC 25922	S.Aureus ATCC 25923
	Unit 1	Unit 1
HI-3	Nil	Nil
Hdrg1 (24μg)	20mm	12mm
Gentamicin (10 μg)	20 ±1.5mm	33±0.5mm

Table 11. Zone of inhibition of H (negative control), Hdrg and Gentamicin (positive control) against E.*Coli* and S.aureus

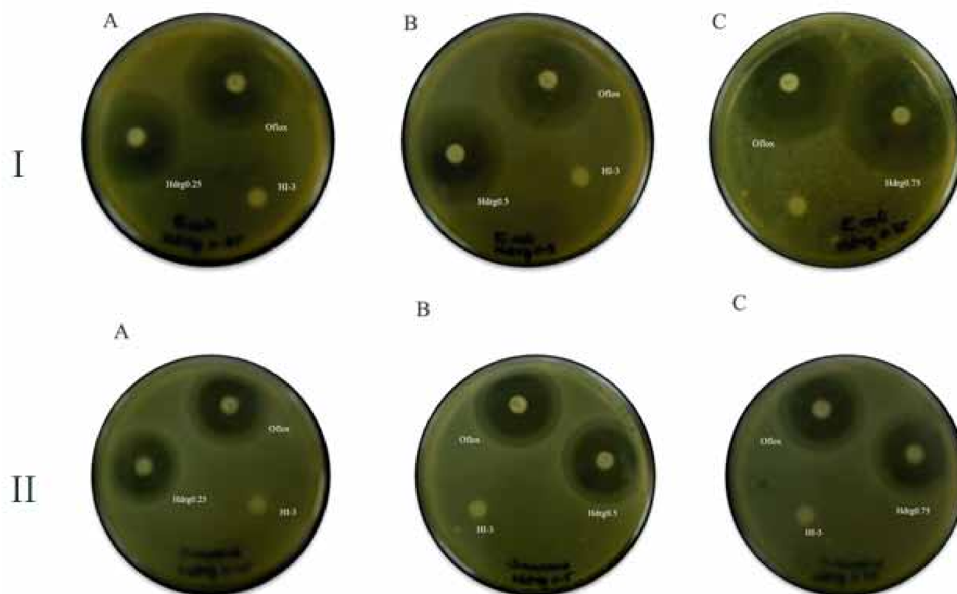

Figure 50. Representative patterns of zone of inhibition assay of (A)Hdrg-0.25 (B)Hdrg- 0.5(C)Hdrg-0.75 against (I) *Escherichia Coli.* (II) *Staphylococcus aureus*

Test material code	*E. coli* ATCC 25922	
	Zone of inhibition (H)=(D-d)	Growth of bacteria under the specimen
HI-3 (negative control)	0	Heavy growth
Hdrg 0.25(6 µg)	24	No growth
Hdrg 0.5(12 µg)	24	No growth
Hdrg 0.75(18 µg)	26	No growth
Positive control (Ofloxacin- 5µg)	25	No growth

Table 12. Values of zone of inhibition of H (negative control of Hdrg-0.25, Hdrg- 0.5 Hdrg-0.75 and ofloxacin (positive control) against *E.Coli*

Test material code	***S. aureus* ATCC 25923**	
	Zone of inhibition (H)=(D-d)	**Growth of bacteria under the specimen**
HI-3 (negative control)	0	Heavy growth
Hdrg 0.25 (6 µg)	19 mm	No growth
Hdrg 0.5(12 µg)	23 mm	No growth
Hdrg 0.75(18 µg)	22 mm	No growth
Positive control (Ofloxacin- 5µg)	23 mm	No growth

Table 13. Values of zone of inhibition of H (negative control of Hdrg0.25, Hdrg 0.5 Hdrg0.75 and ofloxacin (positive control) against *S.aureus*

4.4. Preparation and Characterization of the EGF-loaded hydrogel system (H-EGF)

4.4.1. Preparation of H-EGF

Incorporation of EGF on the surface hydrogel discs (6 mm diameter and 1mm thickness) was accomplished using solvent sorption method. Absorption of EGF on the hydrogel matrix was confirmed using surface morphological analysis.

Figure 51. Lyophilized H-EGF

4.4.2. Characterization of H-EGF

4. 4.2.1. Surface morphology

Environmental scanning electron microscopy was used to analyze the effect of EGF absorption on surface morphology of hydrogel system. Figure 52 shows the ESEM image of surface of bare hydrogel HI-3 and H-EGF in the swollen state. EGF was found to be absorbed effectively on the hydrogel matrix as evidenced by Figure 52B.

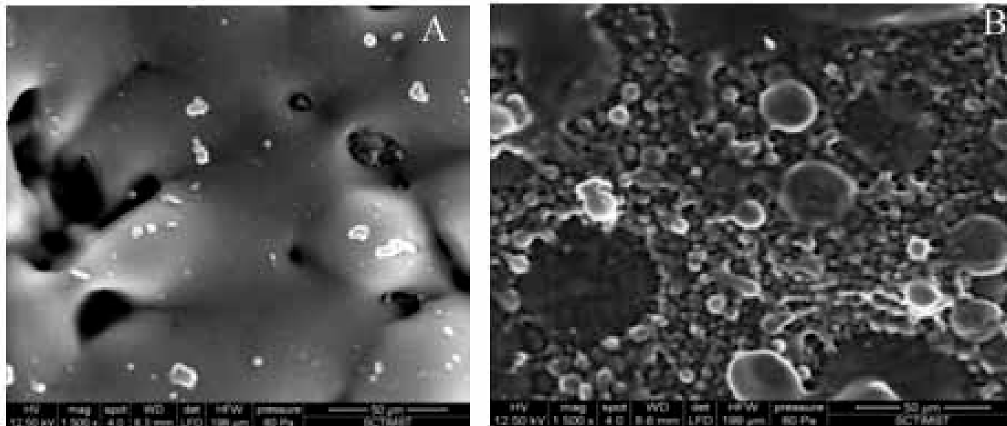

Figure 52. ESEM image of (A) Swollen hydrogel sample HI-1 (B) H-EGF

4. 4.2.2. Surface Wettability Study

Effect of EGF on the hydrophilic property of the hydrogel system was studied using sessile drop method. Contact angle of H-EGF (59 ± 2 ^{o}) indicated presence of EGF provided hydrophobic nature to the hydrogel system.

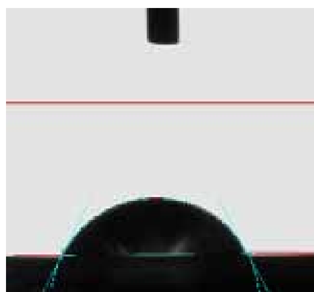

Figure 53. Surface wettability of H-EGF

4. 4.2.3. *In vitro* release profile of EGF

In vitro release of EGF from the hydrogel matrix as a function of time at pH =7.4 is shown in Figure 54. The release profile was analyzed for a period of 140 h. Initial burst release was observed within 24 h. Within 50 h, nearly 100% release was achieved

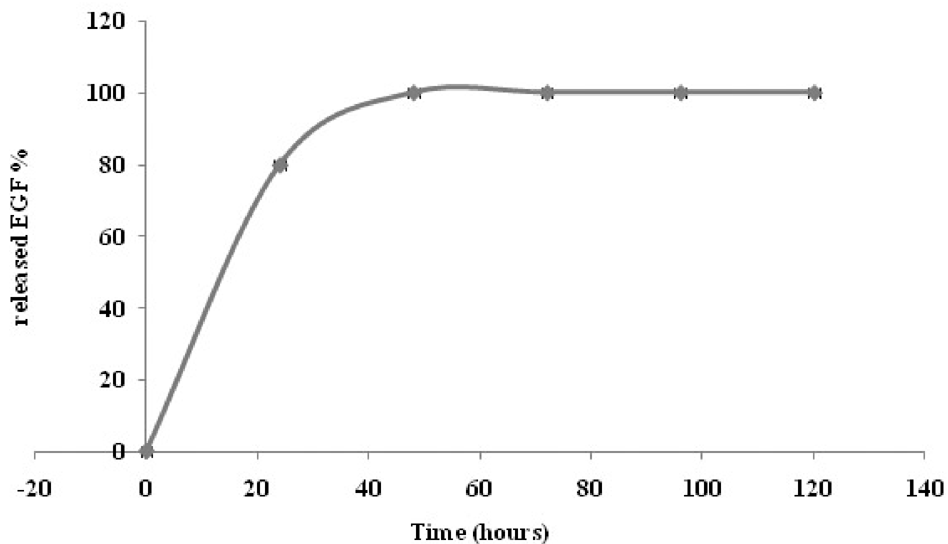

Figure 54. ***In vitro*** **release profile of EGF from H-EGF (n• 3)**

4.4.3. Biological Evaluation of H-EGF

4.4.3.1. Cell Viability Study

Acridine orange and ethidium bromide staining was done to study the cell viability. Figures 55A and B depict the fluorescent microscopic images of control cells and fibroblast incubated with H-EGF. Cell viability of the fibroblast after incubation with H-EGF for 48 h was found to be comparable with that of the control. It was observed that fibroblasts retained nearly100% viability in the presence of H-EGF.

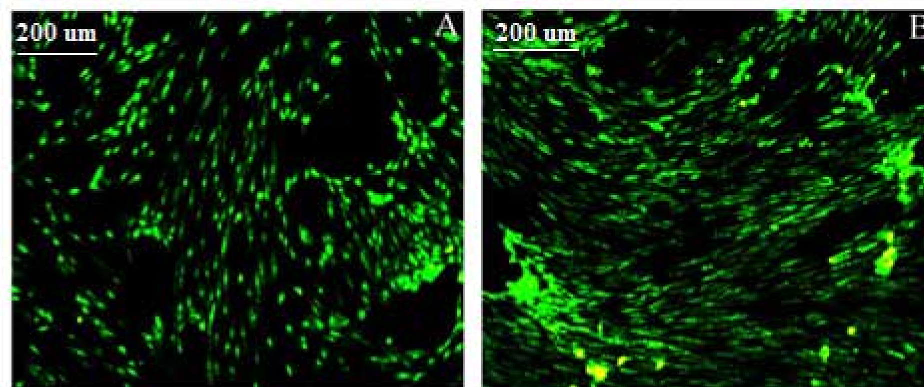

Figure 55. Live-dead assay using acridine orange and ethidium bromide on fibroblast , incubation at 37 ^{o}C for 48 h (A) control (B) H-EGF (n• 3)

4.4.3.2. Cell culture study with H-EGF

Cell culture study was conducted for 24h and 48h to analyze the effect of H-EGF on fibroblast growth. Figures 56(A- C) and (D-F) show the microscopic images of fibroblast obtained after incubation for 24 h and 48 h on the culture plate without the hydrogel matrix, with HI-3 and with H-EGF respectively. It is evident from the images that presence of H-EGF promotes the fibroblast growth in culture when compared to HI-3 as well as with control.

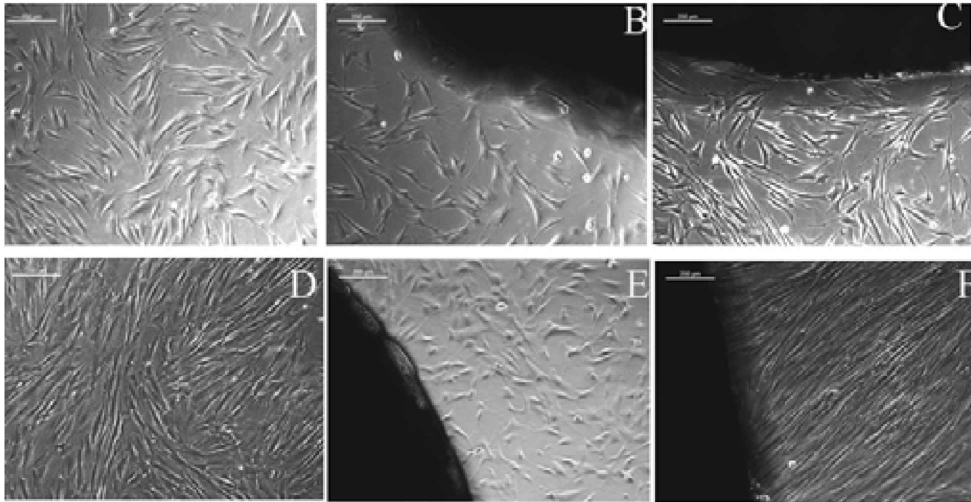

Figure 56. Microscopic images of fibroblast (A) control cells (B) HI-3 (C) H-EGF at 24 h (D) control cells (E) HI 3(F) H-EGF at 48 h (n• 3)

4.4.3.3. Cell Proliferation study - Tritiated Thymidine Uptake Assay

Tritiated thymidine uptake assay of fibroblast was performed to evaluate cell proliferation. Incubation of fibroblast with H-EGF for 48h enhanced the cell proliferation significantly compared to that of the control and HI-3 (Figure 57A). Morphological analysis of cells after incubation with the H-EGF for 48h substantiated the result of quantitative analysis; simultaneously significant decrease in doubling time of fibroblast was observed in the presence of H-EGF (Figure 57B).

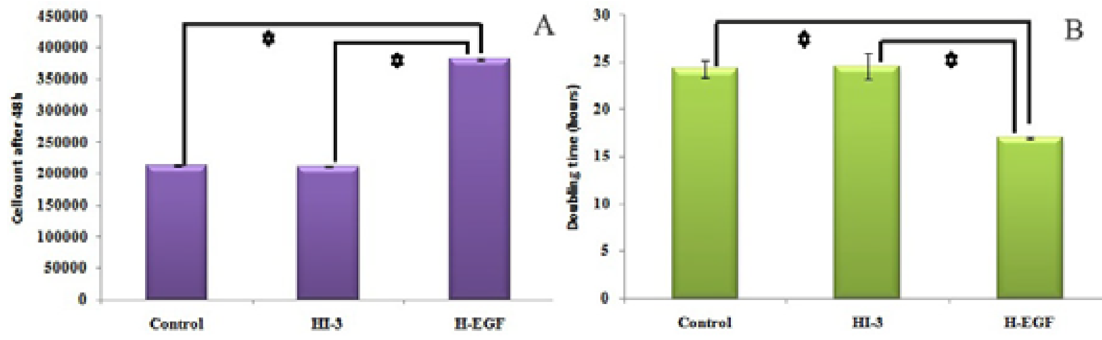

Figure 57. (A) Fibroblast proliferation data (B) Doubling time after incubation with H-EGF for 48h (n• 3)

CHAPTER 5

DISCUSSION

Chapter 5 contains the discussion and interpretation of the study results detailed in Chapter 4. The major findings of this study are correlated with published literature in the relevant field and interpretations made wherever possible. The limitations of the study have been identified and future prospects are outlined.

5.1. Fabrication and characterization of hydrogel matrices

Excellent fluid uptake ability, non-adherence, soothing effect, cooling sensation and biocompatibility are prerequisites for wound dressing materials and among hydrogels, Poly (2-hydroxy ethyl methacrylate) (pHEMA) system has been found to possess good biocompatible and blood compatible properties. The hydrophilic nature of the polymer also imparts a high degree of swelling to the hydrogel systems (Faxalv *et al*., 2010, Singh *et al*., 2010). However, the main disadvantages of pHEMA hydrogels are their low mechanical strength and tear resistance. The problem of poor mechanical strength can be resolved in many ways, especially by incorporating with other polymer systems, adding cross-linking agents or copolymerization. Numerous reports have shown that the incorporation of polycaprolactone, an FDA approved polymer for biomedical applications, improves the mechanical property of hydrogel systems such as poly (vinyl alcohol) and pHEMA(Florence *et al*., 1994). Biocompatibility and blood compatibility of hydrogel systems is also found to improve by modification using poly (ethylene

glycol) (Harrison 1992). Previous work has shown that pHEMA hydrogel tethered with PEG sulphonate exhibits increased swelling and non-thrombogenicity when compared to the pHEMA hydrogel (Jung *et al*., 2006). Hydrogels based on pHEMA and pHEMA-co-PEG have earlier been investigated as wound dressing material (Giglio *et al*., 2011, Rayment *et al*., 2005).

Hydrogels can be prepared using different polymerization techniques. Conventional techniques include redox initiation, ionic initiation and addition mechanisms. However, photo-polymerization has several advantages over conventional techniques. It has spatial as well as temporal control over polymerization, faster rate (less than a second to few minutes) at room or physiological or even higher temperatures and minimum heat generation (Decker, 1987) which is why it was chosen to polymerize the monomer in this study. Photo-polymerization is usually achieved using organic photo-initiators which can be UV or visible light active. The major problem associated with the commonly used photo-initiators is that they tend to impart color to the polymer system. In this study, a novel photo-initiator 2, 4, 6 trimethyl benzoyl diphenyl phosphine oxide (TPO) was selected which facilitated polymerization at a faster rate and did not impart any color to the polymer system. Since the application is for wound dressing, any residual monomer or initiator contact can cause unnecessary toxic problems which was why chemical initiation methods were totally avoided.

Conversion of monomer HEMA to its polymeric form was confirmed by the absence of the infra red absorption peak at 1636 cm^{-1} characteristic of the –C=C- (stretching) chromophore in the FT-IR spectrum for all the hydrogels studied (Figure 21). Characteristic absorption peaks of PCL, pHEMA and PEG in the spectrum were indicated by the absorption peaks at 1725-1730cm^{-1} corresponding to the characteristic -C=O stretching frequency of PCL and pHEMA. Peak at 1163cm^{-1} was indicative of the C-O-C stretching frequency of PEG. The presence of the absorption band at 3533 cm^{-1} indicated the free hydroxyl (-OH) group, which is a characteristic peak of pHEMA. The broad nature of these peaks suggests that hydroxyl groups are extensively hydrogen bonded.

Comparative evaluation of the X-ray diffractogram of the pHEMA system with other hydrogel systems indicated that pHEMA possesses an amorphous character, while the combination hydrogel systems have a semi-crystalline nature (Figure 22). Sharp peaks at 21.40^{o} and 23.71^{o} confirmed the introduction of the crystalline character to the hydrogel systems.

Swelling capacity of the hydrogel network is important, especially for wound dressing application, as it may affect mechanical as well as surface properties (Kopecek *et al*., 2007, Abidian *et al* ., 2006, Omidian *et al*., 2014). The polymer swelling property is a complex process which depends on the nature of polymer, polymer-solvent compatibility and degree of cross-linking (Richard *et al*., 1981). Previous studies reported that the moist environment is more favorable to re-epithelialization compared to the dry environment (Winter, 1962). With its good swelling capacity, the hydrogel system is expected to make

the wound moist, absorb exudates, provide a soothing effect and cooling sensation, and facilitate wound healing. As the prepared hydrogel samples (HI-1 – HI-6) were composed of hydrophilic and hydrophobic units, system HI-3 exhibited maximum swelling of 64 ± 2.8 % and system HI-5 showed minimum swelling of 52 ± 1%. It was found that of all the hydrogel systems, HI-1 and HI-5 had the highest amount (95 parts) of hydrophilic pHEMA. Other components present in HI-1 were 2.5 parts of hydrophilic component PEG and 2.5 parts of hydrophobic component PCL. Reduced swelling of HI-5 might be due to the presence of 5 parts of the hydrophobic component PCL and the absence of hydrophilic PEG. Significant increase in the swelling capacity of HI-1 indicated that HI-1 is more hydrophilic compared to HI-5 due to higher pHEMA content. However the swelling ability of HI -3 (85 parts HEMA, 10 parts PEG and 5 parts PCL) was significantly higher than HI-1, which indicated that incorporation of PEG enhances the hydrophilic nature considerably.

The mechanical properties of a wound dressing material are important as the dressing should protect the wound in the absence of skin at the site. The dressing material should remain intact and conform to the body and wound surface (Queen *et al*., 1986). To ensure proper protection, it should be mechanically strong enough to resist tearing and sufficiently elastic to accommodate proper movement. Poor mechanical properties of hydrogel in their swollen state can be improved by addition of PCL (Mohan *et al*., 2008, Florence et *al*., 1994). Natural skin is reported to have a tensile strength of 5-30 MPa and tensile strain of 35-115%. Though the tensile strain of HI-3 was found to match with that of normal skin, the strength values were expectedly low as the material developed were

hydrogel systems with high water absorbent capability and is expected to function as a temporary wound dressing material only for short periods but had enough handling characteristics for placement over wound structure. As the stress- strain properties, swelling and surface wettability were superior for HI-3 hydrogel, it was selected for further studies.

Water wettability of hydrogel systems is an important feature which affects their performance and biocompatibility (Hirota *et al*., 2006, Arica *et al*., 2005). PHEMA and PEG being totally hydrophilic, the incorporation of PCL tends to introduce hydrophobic character into the matrix to varying degrees (Jones *et al*., 2001, Jones *et al*., 2002). Hydrogel system HI-3 was found to be less hydrophilic than pHEMA due to the presence of 10% PCL in the matrix.

Figure 27 shows the TGA thermogram of lyophilized hydrogel matrix. It indicated that the hydrogel system is thermally stable up to 196 ^{o}C. Figure 28 depicts the DSC thermogram of the hydrogel system, from which it is clear that HI-3 contains only one glass transition temperature at -27.8 ^{o}C. The single glass transition temperature of the system indicates that the components in the blend are completely miscible, which supports the observation of Brostow *et al* that full miscibility of blends is characterized by a single glass transition temperature (Brostow *et al*., 2008). The onset of melting is observed at 51.85 ^{o}C.

Surface analysis of the HI-3 hydrogel revealed two different types of (Figures 29a&b) surface morphology. The outer surface is seen to be composed of a dense skin

layer and a porous inner layer structure. The dense outer layer provided a protective barrier and controlled water loss through evaporation and excessive loss of body fluid, and provided physical protection from the environment. The porous inner layer is expected to help in the promotion of drainage, prevention of exudates buildup and preparation of an optimal wound bed for auto-grafting (Kim *et al*., 2000).

An ideal wound dressing material is one that would control water loss from the wound in order to facilitate proper wound healing (Balakrishnan *et al*., 2005). The reported values of evaporative water loss for normal skin are 204 ± 12 g/m^2/day, 279 ± 26 g/m^2/day for a first degree burn, and 5138 ± 202 g/m^2/day for a granulating wound (Lamke *et al*., 1977). Wound dressing material should be permeable to water vapor in order to prevent excessive wound dehydration and simultaneous removal of wound exudates. It has been recommended that a mid-range water loss rate of 2000-2500 g/m^2 /day from injured skin would suffice for a wound dressing to provide optimum moisture to the wound (Mi *et al*., 2001). Some of the commercially available hydrogel-based wound dressings such as Geliperm (Geistlich Ltd., Switzerland) and Vigilon (Bard Ltd., Crawley, UK) were reported to have a water vapor permeability rate of 9009 ± 319 g/m^2/day and 9360 ± 34 g/m^2/day respectively (Wu *et al* .,1995). The higher water permeability of these dressing materials causes wound dehydration and adherence of the dressing to the wound, and ends up in complications such as infection and bleeding. Hydrogel system HI-3 in the present study showed a value of 2010 ± 100 g/m^2/day, which is expected to provide an optimum fluid balance at the wound site to facilitate proper wound healing.

5.1.1. Biological Evaluation of Hydrogel Matrix

In vitro hemolysis testing is the most reliable method for determining the hemocompatibility of materials which come into contact with blood (Balan *et al*., 2014). Zhou *et al* reported that, for a biomaterial to be non-hemolytic, the percentage hemolysis should be less than 5% (Zhou *et al*., 2011). HI-3 was found to be compliant with this requirement and showed non hemolytic character as it generated only a hemolysis of $0.8 \pm 0.1\%$.

Data revealed (Figures 30 A-B) HI-3 to be non-cytotoxic when compared with the control (fibroblasts grown on culture plate). The cells were found to retain their characteristic spindle-shaped morphology after 48 h in contact with the HI-3 hydrogel. Cell viability study using live/dead assay indicated 100% cell viability in presence of HI-3 which validated the non-cytotoxic nature of the hydrogel matrix (Figures 31 A&B). Several reports have shown that pHEMA hydrogel does not induce any cytotoxic effect to biologic systems (Cifkova *et al*., 1988, Korbelar *et al*., 1988). An ideal wound covering material is expected not to cause cell adhesion as this would cause pain upon the removal of the dressing from the wound. Actin staining was carried out to evaluate the cell adhesion property of hydrogel matrix. Microscopic observation of the cells indicated non–adherence of the cells to the material (Figure 32 B). The incubation of hydrogel matrix HI-3 on the sub-confluent monolayer of fibroblasts for 72h did not produce any unfavorable effect on cell spreading, as evidenced by actin organization and network formation in the cells lying underneath the gel (Figure 32 C). Cell morphology as well as spreading in the medium underneath the gel was found to be comparable to the bare

medium (Figure 32 A). The spindle-shaped morphology of the cells was also prominent, validating the earlier non-cytotoxic character of the hydrogel. The non-cell adhesion property of hydrogel can be explained in terms of its high water content and hydrophilicity, and the low interfacial tension between the hydrogel and the surrounding medium (Li *et al*., 2004).

5.2.Fabrication of SNP incorporated hydrogel matrix (HSN-3)

Poly (ethylene glycol) protected SNP was synthesized and characterized in the laboratory. Various polymeric systems such as poly (N-vinyl pyrrolidone) (PVP), poly (acrylonitrile) (PAN) and cellulose acetate (CA) have proven to be proper protecting agents during silver nano particle synthesis (Yang *et al* .,2003, Son *et al* .,2006, Jin *et al* .,2005). In the present study, PEG acts as a protective agent for silver and simultaneously improves the miscibility of nanoparticle solution with the hydrophobic segment PCL.

Silver nanoparticles could be incorporated successfully into the hydrogel matrix using a photo-polymerization technique (HSN-3, Figure 33). The significant reduction in the swelling capacity of HSN-3 might have been due to the interaction of the silver nanoparticles with the hydroxyl group of pHEMA, which eventually reduced the water absorption capacity of hydrogel system. The contact angle study indicated that the addition of SNP to the hydrogel system could effectively decrease the surface hydrophilicity of the system.

The silver nanoparticle-incorporated hydrogel system HSN showed a sustained release of SNPs from the matrix (Figure 35). It was observed that nearly 75 % of the SNP

was released within 4 days of immersion. It is assumed that the remaining silver nanoparticles (25%) got entrapped within the bulk of the hydrogel matrix. This is expected to help the controlled release of SNPs during the extended period of application.

5.2.1. Biological Evaluation of HSN-3

The hemolytic properties of wound dressing material are important as it comes into contact with blood. % hemolysis assay indicated that SNP incorporation did not produce any significant change in the hemolytic properties of the hydrogel system. The low hemolysis value of 1.6 ±0.3% exhibited by HSN-3 signaled the blood compatible nature of the hydrogel. Since the value falls within the acceptable limit of 5%, HSN-3 is considered to be safe for wound dressing applications.

The released SNPs (~18mg/L) showed no cytotoxic effect on the fibroblast as evidenced by the direct contact assay and live/dead assay (Figures 38 & 39), which substantiated the observation of Zhang *et al* that SNP concentration at or below 25g/L did not induce any cytotoxic effects on the fibroblasts cells (Zhang *et al* .,2005). This concentration is significantly lower than the reported non-cytotoxic concentration and is expected to significantly inhibit the microbial growth. The fibroblasts were found to maintain their spindle-shaped morphology and cell viability after incubation with HSN-3 for 48 h. Tian *et al* reported that the topical delivery of SNP promotes wound healing (Tian *et al*., 2007).

The cell adhesion study carried out for 72 h did not show any unfavorable effects on cell spreading as evidenced by the actin organization and network formation underneath the HSN-3 (Figure 40 F). Microscopic observation of the hydrogel surface in contact with the cells indicated the non-adherence of the fibroblast to the material (Figure 40 E). The non cell adhesive property of HSN-3 was found be similar to that of HI-3. Hydrogel systems inherently possess a non-adherent nature because of their low interfacial tension with the surrounding fluid. In addition to that, the presence of SNPs in the system may also contribute to the non-adhesion of the fibroblasts on its surface.

5.2.2 Evaluation of Antimicrobial Activity of HSN-3

SNPs have been found to be non-toxic, environment-friendly antibacterial material for various biomedical applications. SNPs have long been used for controlling infection in burn patients (Kalsen, 2000). In biomedical applications, SNPs are used in wound management because of their potential to control infection as well as inflammation (Fidel *et al*., 2012). The representative patterns of the zone of inhibition of HSN-3 against both gram-negative and gram-positive strains are shown in Figure 41A and B. Data showed that HSN-3 was found to exhibit antibacterial property against both strains. Other reports on the antibacterial effect of silver nanoparticles validate the antibacterial properties of HSN-3 (Lee *et al*., 2005). To exhibit antibacterial properties, silver nanoparticles must be converted to silver ions via oxidation of silver. Silver ions released from the silver nanoparticles diffuse into the media. Moreover, the hydrogel matrix can provide a suitable media to facilitate diffusion of the hydrated silver ions. Srivastava *et al* reported that SNPs exhibited greater activity against gram-negative bacteria compared to gram

positive (Srivastava *et al*., 2007). The antimicrobial effect of HSN-3 was found to be more pronounced against gram-negative bacteria than gram-positive bacteria which substantiated the previous report of SNPs antimicrobial action (Srivastava *et al*., 2007) . The interaction of the nanoparticles with the cell wall of bacteria could have been facilitated by relative abundance of negative charges on gram negative bacteria, so that the SNPs could easily penetrate into the cells and strongly associate with the cellular components that caused more effective inhibition of bacterial growth (Sui *et al*., 2006).

5.3. Fabrication and Characterization of the ofloxacin-incorporated hydrogel matrix-Hdrg

Various methods have been reported for preparing drug-incorporated hydrogel matrices for biomedical applications (Kim *et al*., 1991). Song *et al* showed that effective incorporation of a drug can be achieved by the incorporation of the drug in the monomer mixture along with the initiator and other additives and then allowing it to polymerize (Song *et al*). In the present study, photo-polymerization was successfully employed for the preparation of Hdrg (Figure 42) by addition of a drug in the monomer and initiator. Ofloxacin has been found to be effective in controlling skin structure infection caused by gram-negative and gram-positive aerobic bacteria (Covino *et al*., 1990, Yuk *et al*., 1991). It is recommended that burn wound infections can be effectively reduced by the application of wound dressings incorporated with antibacterial agents rather than by applying topical ointments. The drug delivery system was developed for the purpose of bringing, taking up, retaining, and releasing the drug in right dose at the site (Langer, 1990, Rathbone *et al*., 1999). Various methods such as dipping or pasting have been adopted for incorporating the antibiotic agent silver sulphadiazine in pHEMA systems to develop antimicrobial burn wound dressings. However, long-term diffusion efficacy and fast elimination of the drug made these techniques ineffective (Robb *et al*., 1981, Fox *et al*., 1980, Fang *et al*., 1987, Lee, 1985, Miller *et al*., 1990). Thermal methods for impregnation and immobilization of antimicrobial agents onto pHEMA membranes have been reported earlier (Nathan *et al*., 1982 , Meslard *et al*., 1986, Tyagi *et al*., 1993). Tsou

et al used a UV irradiation technique to prepare poly (2-hydroxy ethyl methacrylate) wound dressing systems incorporated with ciprofloxacin (Tsou *et al*., 2004).

Figure 43 indicates the percentage swelling of the hydrogel systems. Effect of incorporating the drug on swelling property compared to bare hydrogels can be observed. It was seen that incorporating the drug ofloxacin tends to decrease the swelling rate of the hydrogel significantly. The contact angle study also substantiated this observation. Incorporation of drug in the hydrogel system considerably enhanced its hydrophobicity. These results imply that the addition of the drug to the hydrogel system had an influence on its surface as well as on its bulk properties. The drug encapsulation study indicated that nearly 60% drug was entrapped within the matrix effectively using the photo polymerization method.

In vitro drug release from the matrix was evaluated in PBS (pH =7.4) and water (pH=7) (Figures 44 A & B). Burst release of the drug from the matrix was observed within the first 24 h in PBS compared to that in water. Nearly 85% of the drug was found to be released within 24 h. Ions present in the PBS may have facilitated the burst release of drug from the matrix. Different drug loading on to the hydrogel matrix was done to evaluate its effect on drug release and antimicrobial effect. Figure 45 displays the release profile of drug from hydrogel at different drug loadings. Release data shows that formulations containing the highest amount of the drug displayed faster and higher release rates than those of the formulations with a lower amount of drug loading. The release rate becomes quite slower at the lower amount of drug in the matrix due to the availability of more free void space for the passage of the drug molecules.

The k and n values of the hydrogel systems were determined from the graph driven (Figure 45) via the equation 5and are given in Table 11. When n=0.5, the drug diffuses and is released from the matrix and it tends to obey Fickian diffusion kinetics. For n> 0.5, an anomalous, non-Fickian drug diffusion occurs. When n=1, a non-Fickian, case II or zero order release kinetics could be observed. In the present work, n values of all the hydrogel systems were observed to be greater than 0.5 indicating drug releases to occur through a swelling controlled non- Fickian diffusion kinetics

5.3.1. Biological Evaluation of Hdrg

Hemolytic property evaluation of Hdrg was carried out as per standard ISO 10993-4:2002 (E) and the result indicated that incorporation of ofloxacin in the matrix did not induce any hemolysis since and the % hemolysis (1.56 ± 0.3%) was much below the permissible level of 5 %.*In vitro* cytocompatibility evaluation using the direct contact test indicated that neither the released drug nor the drug within the matrix produced any unfavorable effect on the fibroblast growth. Figure 46C shows the microscopic image of well spread out, healthy spindle-shaped fibroblast in the vicinity of Hdrg1 which are found to be comparable to the fibroblast around HI-3(Figure 46B). Nearly 100% of the fibroblast were found to be viable after incubation with Hdrg1 for 72 h (Figure 47 B). The cell viability assay substantiated the i*n vitro* cyotcompatibility property of Hdrg1. Studies of actin staining indicated that the introduction of drug in the matrix does not produce any change in the non-cell adhesion property of the hydrogel system (Figure 48 H). Cells underneath the hydrogel system were found to maintain their spindle-shaped morphology

with a well spread out nature (Figure 48 I). These results validate the earlier non-cytotoxic property of the Hdrg-1.

5.3.2. Evaluation of Antimicrobial Activity of Hdrg

Due to its broad spectrum of antibacterial activity, ofloxacin was clinically proven to be effective in controlling various infections such as skin and soft tissue infection, respiratory tract infections, urinary tract infections, etc. The antibacterial activity of the hydrogel systems with different amounts of drug loading was evaluated using the agar disc diffusion method. Antibacterial activity of the hydrogel system with various amounts of drug loading was evident, as shown in Tables 11, 12 and 13. Figures 49 and 50 show the clear inhibitory zones exhibited by the Hdrg against Gram-positive and Gram-negative bacteria. The antibacterial activity of Hdrg1 was compared with that of the gentamicin disc (Figure 49 and Table 11). As can be seen from the size of the inhibitory zones (Figure 49A), similar antibacterial activity was exhibited by both the sample and control against the *E.coli* strain. Figure 49 B indicates that the antibacterial activity of Hdrg1 against *S.aureus* was slightly less compared to the control. Comparative evaluation of the antibacterial activity of Hdrg (Hdrg0.25, Hdrg 0. 5 and Hdrg 0.75) with the ofloxacin disc is shown in Figure 50. The zone of inhibition of Hdrg (Hdrg0.25, Hdrg 0. 5 and Hdrg 0.75) was found to be similar to that of the control ofloxacin disc (Tables 12 and 13). All the three Hdrg systems contain a greater amount of the drug compared to that in the control, and the similarity in the antibacterial effects may be due to the difference in the drug release mechanism. The antibacterial effects of Hdrg were found to be more pronounced against Gram-negative bacteria than Gram-positive bacteria. Reports

have proven that ofloxacin exhibits greater inhibitory action against gram-negative bacteria compared to gram-positive bacteria (Monk *et al*., 1987). Sato *et al* reported that ofloxacin is the most effective fluoroquinolone against staphylococci (Sato et al., 1986). The antibacterial activity of the fluoroquinolone has been attributed to the inhibition of the A subunit of the enzyme DNA gyrase, which prevents the super coiling of bacterial DNA. Furthermore, ofloxacin may have an additional inhibitory effect on the B subunits of the enzyme (Monk *et al*., 1987).

5.4. Preparation and characterization of EGF-loaded hydrogel system (H-EGF)

Epidermal growth factor plays an important role in the wound healing mechanism. Various reports have shown that EGF could facilitate epithelialization both *in vitro* and *in vivo*. Natural as well as synthetic polymer systems such as alginate, gelatin, collagen and poly(ethylene-co-vinyl acetate) have been reported as carriers of EGF. But the biggest problem of protein-releasing systems is the loss of activity of the protein that results from their denaturation and deactivation during the formulation process within the matrix. In this context, the solvent sorption method was used to load EGF onto the hydrogel matrix. Dogan *et al* reported solvent sorption as an effective method to load EGF and bFGF on hydrogel systems (Dogan *et al*., 2005). The swelling capacity of the hydrogel system made the EGF loading procedure easy and effective, i.e., the loading was done on dry disc. Previous reports showed that exposure to higher temperature may lead to loss in activity of growth factor. To minimize the activity loss of growth factor, the loading procedure was carried out in PBS at room temperature.

The ESEM photographs (Figures 52A & B) show the difference between the unloaded and loaded hydrogels and also the distribution of the growth factor on the surface of hydrogel. Result of surface morphology analysis indicated that solvent sorption is an effective method for EGF incorporation on to the hydrogel matrix. Water contact angle study indicated that presence of growth factor increased the hydrophobicity of the hydrogel system.

Observation of the matrix showed that *in vitro* release kinetics of EGF was followed by the intrinsic tryptophan fluorescence. Previous studies have shown that

fluorescence spectroscopy is an effective method for the quantitative analysis of proteins (Ladokhin *et al.*, 2000). EGF contains three chromophores in its amino acid sequence, namely, tryptophan, tyrosine and phenylalanine .The fluorescence excitation and emission spectra of EGF before and after the loading procedure showed that the loading procedure does not produce any change in the confirmation of growth factor. These results further indicate that the activity of the growth factor is retained after the loading procedure despite extended contact with the hydrogel and medium. The amount of EGF released from the hydrogel matrix as a function of time at pH =7.4 is shown in Figure 54. The release profile was characterized by an initial burst of protein within 10h followed by sustained release. The initial burst effect is attributed to the localization of the growth factor closer to the surface of the hydrogel during the loading and drying processes. After the embedding procedure, drying of the loaded hydrogel starts at the surface and water from the interior of the hydrogel is pulled to the surface along with the protein by the interaction of the capillary forces in the absence of specific polymer–protein interaction. The mechanism of EGF release is diffusion controlled through aqueous channels within the hydrogel. The water uptake ability of hydrogel promotes controlled delivery of the growth factor over an extended time period.

5.4.1. Biological Evaluation of H-EGF

It was observed that incubation of H-EGF on the subconfluent of monolayer of fibroblast for 48 h did not produce any toxic effects on the cells as evidenced by the cell viability assay carried out using ethidium bromide / acridine orange (Figure 55B) compared to control (Figure 55A). Figure 56 shows the optical microscopic images of fibroblasts

obtained after incubation on polystyrene culture plates in the medium for 48h in the presence of hydrogel without EGF (HI-3) and H-EGF. It appears that after 24h, the fibroblasts cells incubated in the presence of H-EGF exhibited considerably good growth rate (Figure 56 C) compared to those incubated in the presence of HI-3(Figure 56 B). At the end of 48 h of culture, the fibroblast incubated in the presence of H-EGF attained almost 100% confluence as compared to those incubated in the presence of HI-3(Figures 56D-F). Data clearly indicates that H-EGF promotes fibroblast growth considerably, cells show good morphology, more dense and are aligned better. This is further confirmed quantitatively using thymidine uptake assay. The cell proliferation study using the tritiated thymidine uptake assay indicated that the incubation of fibroblast in presence of H-EGF increased fibroblast proliferation significantly compared to control and HI-3 (Figure 57 A). Results showed a significant decrease in doubling time in the presence of H-EGF, indicating faster growth with a healthy morphology (Figure 57 B).

5.5. Limitations of Study

The present study made sure that most of the relevant properties of the hydrogel system required for wound dressing material is evaluated. However, the study lacks evidence on the gas permeability property of the hydrogel matrix, which is an important property of good wound dressing material which could not be evaluated due to limitations.

5.6 Future perspectives

Extension of the work into *in vivo* models will be the final criteria in deciding the potential application of these hydrogels as wound dressings in clinical practice.

CHAPTER 6

SUMMARY AND CONCLUSION

6.1 SUMMARY

The major problems associated with burn wounds are excessive fluid loss and infection. Improper management of wounds is likely to cause life threatening situations. Therefore, proper wound management is required to address these major issues and can be achieved only by using proper wound dressing materials. The selection of appropriate dressing is crucial for optimum healing and for enhancing the quality of life of patient. Research worldwide continues to focus on developing an ideal wound dressing material, but the challenge remains unresolved to a large extent.

The primary objective of the present study was to address the above problem by designing a hydrogel matrix system based on a combination of poly(2-hydroxy ethyl methacrylate)-poly(ε-caprolactone)-poly(ethylene glycol) [pHEMA-PCL-PEG]. Reports have shown that photo-polymerization is an effective method for hydrogel preparation. The fabrication of a polymer hydrogel matrix was successfully accomplished using the photo-initiator 2, 4, 6 trimethyl benzoyl biphenyl phosphine oxide (TPO). TPO was found to be highly effective in completing polymerization within minutes and did not impart any color to the hydrogel system. Chemical characterization of the hydrogel system using FT-IR confirmed the complete polymerization of monomer and the XRD study demonstrated the semi-crystalline nature of the hydrogel in its dry state. Single glass transition

temperature value obtained from thermal analysis study indicated that the components of the system were completely miscible. Among the different compositions of the prepared hydrogels, HI-3 hydrogel with suitable physico-mechanical properties required for a wound dressing material was found to be a promising candidate for further biological study. It's non-cytotoxic, non-hemolytic behavior and non-adherence to fibroblast was expected to support the potential use of this hydrogel system in wound covering applications.

Risk of infection-related mortality is a major concern of burn wounds. Reports have shown that both gram-negative and gram-positive bacteria accumulate in the wound site within 48h of the burn injury. So the development of a wound dressing material with antimicrobial properties is considered essential. Evaluation of the antimicrobial properties and their concentration is crucial since increased dosage of the antimicrobial agent may cause toxicity. In this study, silver nano particles were prepared, characterized and incorporated into the hydrogel matrix successfully using photo-polymerization. It was found that photo polymerization did not influence the antimicrobial activity of silver nano particles incorporated in the matrix. Incorporation of silver nano particles was found to retard the fluid uptake ability of the hydrogel, while the surface hydrophilicity showed improvement. Sustained release of SNPs was observed from the hydrogel matrix. The zone of inhibition assay using the disc diffusion method demonstrated the anti-microbial behavior of HSN-3 against both gram-negative and gram-positive bacteria. Incorporation of SNPs was not found to elicit any cytotoxic response on the fibroblast during *in vitro* studies. It is also demonstrated that HSN-3 does not cause any lysis to the blood while

maintaining antimicrobial property and fibroblasts did not adhere to the hydrogel; favoring its use as a potential candidate for burn wound dressing applications.

Ofloxacin is found to be an effective antibiotic to control burn wound infection clinically. In the present study, photo-polymerization technique could be adopted successfully for fabricating the antibiotic incorporated hydrogel system and nearly 60% of the drug could be effectively encapsulated within the matrix. Incorporation of the drug was found to decrease the water uptake ability of hydrogel significantly. The contact angle study revealed that presence of the drug tends to increase the hydrophobicity of the hydrogel system. The *in-vitro* drug release study showed that release rate is faster when the drug-loading capacity is high. Drug release kinetics indicated that drug release from the matrix follows an anomalous, non-Fickian drug diffusion kinetics. Biological evaluation demonstrated the non-cytotoxic, non-hemolytic behavior of Hdrg and its non-adherence to the fibroblast. Antimicrobial property evaluation showed that Hdrg exhibited a comparative zone of inhibition against both gram-negative and gram-positive bacteria to gentamicin as well as ofloxacin control.

Epidermal growth factor (EGF) plays an important role in wound healing. EGF could be successfully loaded on the hydrogel matrix using the solvent sorption method. Environmental scanning microscopy analysis confirmed the absorption of EGF on the hydrogel surface. The contact angle study indicated that the presence of EGF in the hydrogel decreases the surface hydrophilicity of hydrogel. The *in vitro* release study using fluorescence spectroscopy demonstrated nearly 100% EGF release within 48 h. The tritiated thymidine assay indicated that incubation of fibroblast in the presence of H-EGF

increased fibroblast proliferation significantly compared to the control and HI-3. Simultaneously, H-EGF reduced the doubling time of the fibroblasts significantly compared to the control and HI-3.

Overall, the present study covered wound management on the basis of four different aspects: wound management using a hydrogel dressing to act as a wound covering material; feasibility of two types of antimicrobial hydrogel dressing; protection of the wound from infection with dressing material incorporated with (1) SNPs and (2) antibiotic ofloxacin, and the suitability of hydrogel dressing with EGF to promote wound healing.

6.2 CONCLUSIONS

- Formation of a semi-crystalline hydrogel by photopolymerization of HEMA monomer incorporated with PCL and PEG at 410nm was confirmed by FT-IR and XRD.
- TPO was found to be an efficient, colorless and non-contaminating photo-initiator for the above system.
- The synthesized hydrogel system was found to have physical and chemical properties matching the requirements of temporary wound dressing materials.
- The non-cytotoxic, non-hemolytic nature of the HI-3 hydrogel system and its non-adherence to fibroblast substantiated its selection as a potential wound dressing material

- Silver nanoparticle incorporation was found to induce antimicrobial characteristics in the hydrogel system.
- Ofloxacin-incorporated hydrogel system with 60% drug encapsulation efficiency was developed and the system exhibited excellent antimicrobial character, non-cytotoxicity and non-adherence to fibroblast.
- EGF loading on the hydrogel surface could be accomplished using the solvent sorption method.
- The tritiated thymidine incorporation assay confirmed fibroblast proliferation in the presence of H-EGF and simultaneous reduction in the doubling time.
- Hydrogel systems based on pHEMA-PCL-PEG were developed successfully with potential application as a short-term burn wound dressing material.
- pHEMA-PCL-PEG hydrogel systems incorporated with silver nanoparticle and antibiotic ofloxacin respectively are expected to control infection at the burn wound site.
- pHEMA-PCL-PEG hydrogel incorporated with EGF is proposed for burn wounds where delayed wound healing is expected.

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LIST OF PUBLICATIONS

Original Papers

1. P K Rethikala and Krishnan.V Kalliyana ‘Photo-polymerized poly (2-hydroxyethyl methacrylate) / PCL / PEG system as a potential wound dressing material’ Journal of bioactive and compatible polymers 2015, 30(1) , 74-86 (IF 2.5)

2.Rethikala P K ,Renjith P Nair,Lissy K Krishnan and Kalliyanakrisnan V ‘In vitro drug release study and antimicrobial evaluation of Ofloxacin incorporated pHEMA based hydrogels as wound dressing material’ ((Journal of Drug Delivery & Therapeutics 2017, 7(1) , 13-20 (IF 0.6))

Patent

1. An Antimicrobial Wound Dressing Material Kalliyana Krishnan.V., Lissy K.Krishnan and Rethikala P.K. Indian Patent Application No: 201641026539 of 3.8.2016

2.Epidermal growth factor incorporated hydrogel system for wound dressing application Kalliyana Krishnan.V., Lissy K.Krishnan and Rethikala P.K.(Patent application being processed)

Paper Presentation

1.Rethikala P K and Kalliyana Krishnan V 'Fabrication andcharacterization of photocrosslinkable poly (2-hydroxyethyl methacrylate) based hydrogels for wound dressing applications' an international conference FAPS – MACRO, May 15-18, 2013 organized by Indian Institute of Science, Bangalore

2. Rethikala P K and Kalliyana Krishnan V 'Fabrication and characterization of Poly (2-hydroxypropyl methacrylate) Based Hydrogels For Wound Dressing Applications' National seminar on Biopolymers & Green Composites (BPGC 2013), Kalamassery, September 27, 2013

3. Rethikala P K and Kalliyana Krishnan V 'Studies on Development of Novel Polymer Scaffolds for Skin Tissue Engineering Application' National seminar on Polymers in Medicine and Biology, at Bannari Amman Institute of Technology, Coimbatore, 7 – 8 January 2011

CURRICULUM VITAE

Education

Ph.D.Scholar (January 2010- present) at Sree Chitra Tirunal Institute for Medical Sciences and Technology ,BioMedical Technology Wing,Thiruvananthapuram,Kerala,India

Master of Philosophy in Organic Chemistry (2005 July -2007 July), School of Chemical Sciences, Mahatma Gandhi University, Kottayam, Kerala, India (Passed with First Class)

Master of Science Physical Chemistry (2007 November -2008 December), School of Chemical Sciences,Mahatma Gandhi University ,Kottayam, Kerala ,India (Passed with First Class)

Academic Project

M.Sc project at the Department of Inorganic and Physical Chemistry, Indian Institute Of Science, Bangalore, from February -June 2007, under the supervision of Prof. S Umapathy , in the field of computational chemistry and the thesis was entitled as 'Computational studies of Vibrational Circular Dichroism of Chiral Molecules'.

M.Phil project at the School of Chemical Sciences, Mahatma Gandhi University,Kottayam, 2007-2008,under the supervision of Dr.I.Ibnusaud, in the field of Organic synthesis and the thesis was entitled as 'Synthesis of chiral calixarenes based on(2S,3S)- tetrahydro -3-hydroxy-5-oxo-2,3-furandicarboxylicacid'

APPENDIX

PBS (1000ml) pH7.4	
NaCl	-8g
KCl	-0.2g
Na_2HPO_4	-1.44g
KH_2PO_4	-0.24g

(Added distilled water to a final volume of 1000 ml, solution is filtered and stored at room temperature)